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ANTICOAGULANT EFFECT OF INCUBATED FIBRINOGEN[1]

D. C. TRIANTAPHYLLOPOULOS[2]

Abstract

Sterile fibrinogen rendered non-clottable by incubation was mixed with fresh plasma and the thrombin time determined. An appreciable prolongation was observed. The incubated fibrinogen was then fractionally precipitated with ammonium sulphate. The material precipitated between 25 and 50% ammonium sulphate saturation, when added to freshly drawn but still unclotted blood, or native plasma, prevented its coagulation. This action could be reversed by an approximately fivefold dilution with distilled water and addition of calcium chloride and thrombin, thus excluding fibrinolysis as the cause of the anticoagulant effect. Determinations of the respective coagulation factors showed that no decrease occurred in prothrombin, factor VII, plasma thromboplastin component, and fibrinogen. On the other hand a statistically significant decrease in factor V was observed when calcium was present.

Introduction

It was initially observed (11) that incubated plasma when added to fresh plasma appreciably prolongs the thrombin time of the latter. On the other hand experiments to be described in this paper showed that incubated serum lacks this property. These observations suggested that the prolongation of thrombin time is due to a substance present in the plasma but not in the serum and prompted us to investigate the effect of incubated fibrinogen on blood coagulation.

Experimental

1. Blood samples were obtained from nurses, hospital house staff, and patients of the orthopedic wards.

2. Native plasma (5, 10) was prepared from blood collected with siliconized syringes and needles in siliconized glass or polyethylene tubes and kept in an ice bath. The blood was centrifuged for 20 minutes at $2500 \times g$ at 4° C. in a Servall refrigerated centrifuge, type SS-1. The plasma was aspirated with a siliconized pipette, transferred into polyethylene tubes immersed in an ice bath, and used immediately.

[1]Manuscript received October 23, 1957.
Contribution from the Department of Medicine, University of Alberta, Edmonton, Alberta. Supported by a grant of the National Research Council. Presented at the January 1957 11th Annual Meeting of the Western Regional Group, Division of Medical Research, National Research Council, Banff, Alberta. Proceedings.
[2]Present address: Department of Physiology and Pharmacology, University of Alberta, Edmonton, Alberta.

3. Two preparations of fibrinogen were used:

I. Human fibrinogen precipitated from oxalated plasma by half saturation with sodium chloride. The precipitate was dissolved in distilled water and the salting-out process repeated. The new precipitate was redissolved in distilled water equal to half the volume of the original plasma, and was dialyzed against normal saline. All these procedures were carried out at room temperature. The mean value of clottable tyrosine of 12 different preparations of fibrinogen was 89.6% (S.D. 3.17). The experiments were done with 14 different preparations of fibrinogen.

II. A few experiments were done with fibrinogen prepared by the method of Cohn.*

4. The following method was used to incubate and fractionate fibrinogen: Since neither preparation of fibrinogen was sterile, each was passed through a Seitz filter and incubated under sterile conditions. Every 24 hours, samples were taken and tested for coagulability by adding calcium chloride and thrombin. Approximately seventy-two hours' incubation was necessary to render the sodium chloride precipitated fibrinogen incoagulable while the Cutter Laboratories preparation needed only 24 hours. As soon as the fibrinogen preparations ceased to clot, a saturated solution of ammonium sulphate was added until 25% saturation was reached. The negligible precipitate formed after the addition of ammonium sulphate was removed by centrifugation at 20° C. The saturation of the supernatant was increased to 50%. The voluminous precipitate formed was removed by centrifugation and the supernatant discarded. The last precipitate was dissolved in normal saline equal to approximately one-sixth the volume of the original plasma. The solution was dialyzed overnight against saline until the dialyzate remained clear upon addition of barium chloride.

5. Barbital–acetate buffer (4) was prepared by dissolving 0.971 g. sodium acetate and 1.471 g. sodium barbital in 50 ml. distilled water. An aliquot of 25 ml. was mixed with 20 ml. 4.25% sodium chloride, 21.7 ml. 0.1 *N* hydrochloric acid, and 68.3 ml. distilled water. The pH of this solution was 7.35.

6. Thrombin Topical of Parke, Davis, and Company was used. The dry thrombin containing 10,000 N.I.H. units was dissolved in 5 ml. saline. An equal volume of glycerol was added to prevent freezing during storage at −20° C. This preparation was diluted 1:100 with distilled water immediately before use. One-tenth milliliter of this diluted solution containing 1 N.I.H. (National Institute of Health) unit clotted 0.2 ml. fresh oxalated plasma in 7 seconds (5).

7. Thrombin time (5) was measured by the clotting time of a mixture of 0.1 ml. of the diluted solution of thrombin and 0.2 ml. of the solution to be tested. The results were read from a thrombin dilution curve and converted to N.I.H. units.

8. One stage prothrombin time (5), factor V "labile factor, proaccelerin, Ac-globulin" (10), factor VII "pro-S.P.C.A., proconvertin, stable factor" (4), and fibrinogen (5) were determined according to established methods.

*Kindly supplied by Cutter Laboratories.

9. The thromboplastin generation test (1) was performed using tricalcium phosphate adsorbed oxalated plasma instead of alumina plasma (10).

10. Prothrombin consumption was determined by the adsorption and elution technique (7). The residual prothrombin and factor VII of the serum were adsorbed on tricalcium phosphate. Two-tenths molar sodium citrate was used for elution. The eluate was mixed with adsorbed rabbit plasma and the one-stage prothrombin time of the mixture determined.

Presentation and Analysis of Data

1. Comparative Effect of Incubated Plasma and Serum on the Thrombin Time of Fresh Plasma or Fibrinogen

Three milliliters of citrated plasma or serum obtained from the same individual was incubated in tubes 1.4×10 cm. for 48 hours. A third tube containing 3 ml. saline was used as control. Six milliliters of citrated, sodium chloride precipitated fibrinogen or citrated plasma was then added to each of these tubes. After the pH of the test solutions was reduced to that of the saline control with a dilute solution of hydrochloric acid, the thrombin time was determined. Table I shows that only incubated plasma had a thrombin time prolonging effect. Serum lacks this property.

TABLE I

COMPARATIVE EFFECT OF INCUBATED PLASMA AND SERUM ON THE THROMBIN TIME OF FIBRINOGEN OR FRESH CITRATED PLASMA

	Thrombin time (seconds)		
	Saline	Plasma	Serum
Fibrinogen	6.0 (1.2)*	18.0 (0.3)	6.5 (1.08)
Fibrinogen	7.0 (1.0)	9.0 (0.74)	6.5 (1.08)
Citrated plasma	7.0 (1.0)	11.6 (0.52)	6.5 (1.08)
Fibrinogen	8.0 (0.85)	12.5 (0.47)	7.0 (1.0)

*Figures in parenthesis express thrombin activity in N.I.H. units.

2. Effect of Incubated Fibrinogen on Thrombin Time of Plasma

I. Relationship of Length of Incubation of Fibrinogen to Thrombin Time of Plasma

A 0.25% solution of sodium chloride precipitated fibrinogen was incubated at 37° C. for 4 days under sterile conditions. Each day an aliquot was removed and stored frozen. At the end of the incubation period the pH of each sample was determined and found to be uniformly 6.6. One volume of each sample was mixed with two volumes of the same citrated plasma and the thrombin time of the mixture determined. The results have been converted to N.I.H. units of thrombin activity for the sake of clarity and plotted in Fig. 1. The data indicate that incubated fibrinogen has a thrombin time prolonging effect and that this effect is most pronounced after 48 hours' incubation. In the

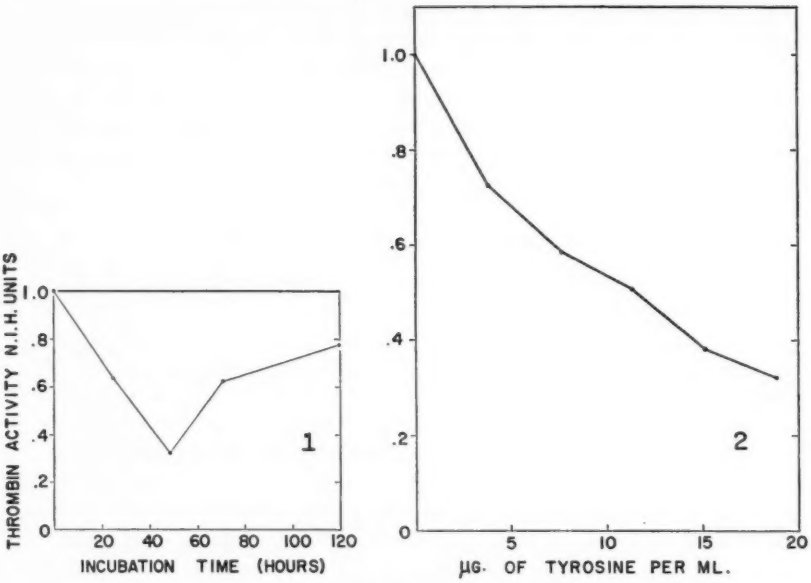

FIG. 1. Effect of fibrinogen incubated for varying lengths of time on the thrombin time of fresh plasma. Sodium chloride precipitated fibrinogen was incubated under sterile conditions for 3 days. Each day samples were taken and kept frozen. On the fourth day of incubation all samples were mixed 1:2 with the same fresh citrated plasma. The thrombin time was read against a thrombin dilution curve and is expressed in N.I.H. units.

FIG. 2. Thrombin time prolonging effect of 24 hour incubated fibrinogen. Three milliliters of fresh citrated plasma was put in each of six tubes. Into each tube increasing amounts of incubated, sodium chloride precipitated fibrinogen were added and the volume brought to 6 ml. with saline. The thrombin time was then determined. The concentration of incubated fibrinogen is expressed in micrograms of tyrosine per milliliter of mixture. The thrombin time was read against a thrombin dilution curve and is expressed in N.I.H. units.

representative example given the thrombin time at zero time was 7 seconds (1 N.I.H. unit), but after 48 hours' incubation became 17 seconds (0.32 N.I.H. unit).

All samples of incubated fibrinogen were tested for their coagulability. Each of them was diluted 1:4 with distilled water, after which calcium chloride and thrombin were added to a concentration of 5 m*M* and 20 N.I.H. units per ml. respectively. The 24 hour sample clotted. The 48 hour sample became slightly opalescent, but no clot was visible. The 72 and 120 hour samples remained clear.

II. Relationship of Concentration of Incubated Fibrinogen to Thrombin Time of Plasma

Three milliliters of fresh citrated plasma was put into each of six tubes. Into each tube increasing amounts of incubated sterile sodium chloride precipitated fibrinogen were added (0, 0.6, 1.2, 1.8, 2.4, and 3 ml.) and the volume

was brought to 6 ml. with normal saline. The pH of all mixtures was adjusted to 7.5. The thrombin time was then determined. The results are plotted in Fig. 2. The concentration of incubated fibrinogen is expressed in micrograms of tyrosine per ml. of mixture. The thrombin time was read against a thrombin dilution curve and is expressed in N.I.H. units of thrombin activity. It can be seen that the addition of one volume of incubated fibrinogen (19 micrograms of tyrosine equivalent per ml. of mixture) to one volume of plasma reduced the thrombin activity from one N.I.H. unit (thrombin time 7 seconds) to 0.33 unit (thrombin time 16.5 seconds).

3. Concentration of Anticoagulant Fraction of Incubated Fibrinogen. Effect on Thrombin Time of Fresh Plasma

Seegers *et al.* (8) noted that fibrinogen becomes incoagulable after standing for a few days at room temperature. This altered fibrinogen cannot be precipitated by 25% saturation with ammonium sulphate. A voluminous sediment, however, is formed between 25 and 50% saturation, which on electrophoretic analysis is found to consist mainly of a single component. The phenomenon was attributed by Seegers *et al.* to contamination with fibrinolysin and is similar to that observed by Garner and Tillett (2). These findings prompted us to study the fraction of incubated fibrinogen which is precipitated by 25–50% saturated ammonium sulphate.*

This fraction obtained either from sodium chloride precipitated fibrinogen or from fibrinogen of Cutter Laboratories was found to inhibit coagulation of citrated plasma by thrombin. Fig. 3 shows such an experiment with the

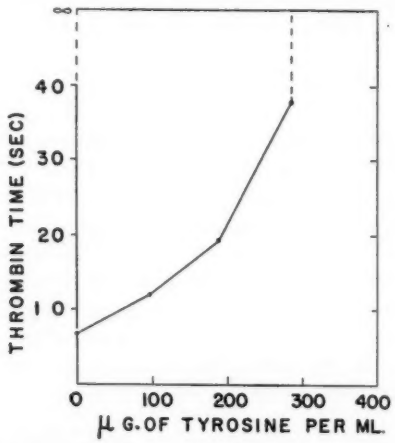

FIG. 3. Effect of incubated "fibrinogen fraction" on thrombin time of fresh plasma. Increasing amounts of Cutter's fibrinogen fraction were added to citrated plasma and the volume completed with saline. The thrombin time was determined. The concentration of the fraction is expressed in micrograms of tyrosine per milliliter of mixture.

*Throughout this paper the precipitate, obtained between 25 and 50% saturation with ammonium sulphate, of a solution of incubated fibrinogen, is referred to as fibrinogen fraction.

"fibrinogen fraction" obtained from Cutter's fibrinogen incubated for 98 hours. Increasing amounts of the fraction were added to citrated plasma, and the volume was completed with saline. The pH of all mixtures was adjusted to 7.5 and the thrombin time determined. The concentration of the fraction is expressed in micrograms of tyrosine per ml. of mixture. The prolongation of thrombin time parallels the addition of increasing amounts of the "fibrinogen fraction". At a concentration higher than 288 micrograms per ml. of mixture, thrombin was unable to clot fibrinogen.

4. Anticoagulant Effect of the Fibrinogen Fraction on Blood or Native Plasma

"Fibrinogen fraction" mixed with native plasma† or freshly drawn plain blood completely prevented clotting at 37° C. for at least 24 hours. The concentration required to inhibit coagulation was found, in seven experiments, to be 45.7 mg. per 100 ml. of plasma–"fibrinogen fraction" mixture (S. D. 17.3), expressed as tyrosine. This action could be reversed by approximately fivefold dilution of the mixture with distilled water and addition of calcium chloride (5 m*M*) and thrombin (50 N.I.H. units per ml.), clotting occuring within 20 seconds.

Fig. 4 shows a representative example. The first tube contains 1.1 ml. "fibrinogen fraction" from Cutter's fibrinogen plus 1.9 ml. blood added immediately after venipuncture (no anticoagulant used). The second tube contains 1.1 ml. normal saline plus 1.9 ml. of the same blood. Both tubes were left at room temperature for one and a half hours and then were photographed. The first tube shows a rapid sedimentation of the red cells and no sign of clotting. The control tube clotted and the clot retracted. The clottable fibrinogen content of the mixture in the first tube was found to be 316 mg. per 100 ml. of mixture. The third tube contains 1 ml. of a 1:1 mixture of sodium chloride precipitated "fibrinogen fraction" with native plasma. The mixture after an incubation of 2 hours at 37° C. did not show signs of fibrin formation. After the addition, however, of 2.5 ml. 0.01 *M* calcium chloride and 50 N.I.H. units of thrombin a clot was formed within 20 seconds.

The clottable fibrinogen content of completely fluid mixtures of blood or native plasma with incubated fibrinogen or its fraction was determined (Table II). After 1–2 hours' incubation at 37° C., 1 ml. of the mixture was diluted with 25 ml. distilled water. To this calcium chloride and thrombin were added to a concentration of 10 m*M* and 10 N.I.H. units per ml. respectively. A clot was formed and its tyrosine content determined. The mean of eight determinations was 184.6 mg. clottable fibrinogen per 100 ml. of mixture. This excludes lack of clottable fibrinogen as the cause of incoagulability.

5. Evidence that Thrombin is not Destroyed by the Fibrinogen Fraction

In order to determine whether the "fibrinogen fraction" destroys thrombin, 50 N.I.H. units of thrombin were incubated with 1.5 ml. buffered "fibrinogen fraction" for 30 minutes. At the end of the period 8.5 ml. distilled water

†Plasma without anticoagulant, obtained with siliconized equipment and kept at 0° C. in polyethylene tubes.

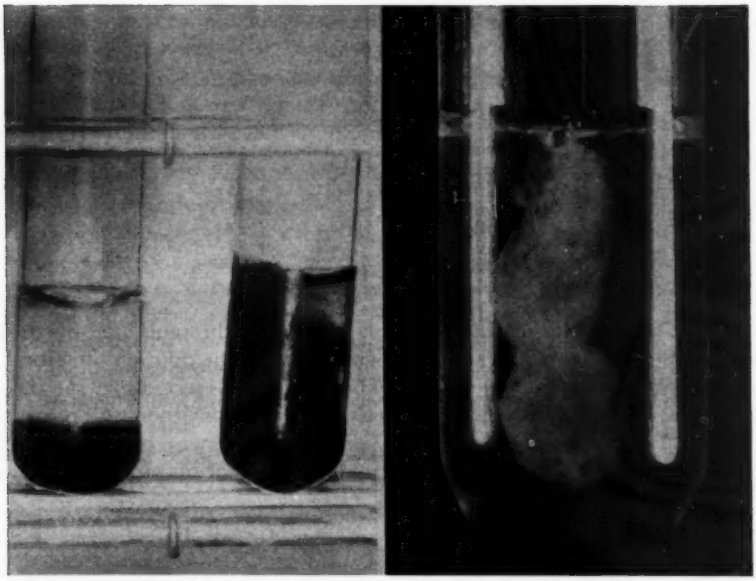

FIG. 4. Anticoagulant effect of "fibrinogen fraction". The first and second tube contain 1.1 ml. Cutter's "fibrinogen fraction" or saline respectively mixed with 1.9 ml. blood containing no anticoagulant. Both tubes were left at room temperature for one and a half hours and then were photographed. The first tube shows a rapid sedimentation of the red cells and no sign of clotting. The control tube clotted and the clot retracted. The third tube contains 1 ml. of a 1:1 mixture of the fraction of sodium chloride precipitated fibrinogen with native plasma. The mixture did not show signs of clotting after an incubation of 2 hours at 37° C. Two and a half ml. 0.01 *M* calcium chloride and 50 N.I.H. units of thrombin were added. After 20 seconds a clot formed.

TABLE II

CLOTTABLE FIBRINOGEN CONTENT OF MIXTURES OF BLOOD OR NATIVE PLASMA WITH INCUBATED FIBRINOGEN OR ITS FRACTION

Mixtures	Clottable fibrinogen in mg. per 100 ml. mixture
One volume Cutter's fibrinogen fraction mixed with three volumes blood	315.0
Three volumes sodium chloride precipitated fibrinogen fraction mixed with two volumes native plasma	166.0
One volume sodium chloride precipitated fibrinogen fraction mixed with 1.7 volumes blood	91.0
4.3 ml. sodium chloride precipitated fibrinogen fraction mixed with 5.1 ml. blood	85.6
One volume sodium chloride precipitated fibrinogen fraction mixed with one volume blood	128.4
One volume sodium chloride precipitated Cutter's fibrinogen mixed with one volume native plasma	150.0
One volume Cutter's fibrinogen fraction mixed with one volume native plasma	208.6
Two volumes sodium chloride precipitated Cutter's fibrinogen mixed with three volumes blood	332.0

TABLE III

EFFECT OF FIBRINOGEN FRACTION ON THROMBIN

Thrombin time expressed as thrombin activity in N.I.H. units

Saline control	Fibrinogen fraction
1.0	1.15
1.0	1.15
1.0	1.79
1.0	1.33
1.0	1.30

was added. This solution was used to determine the thrombin time of citrated plasma (Table III). The results were corrected for the dilution of thrombin. Fractions obtained from five different fibrinogens were tested and the thrombin time was always appreciably shorter (1–3 seconds) than the respective buffered saline controls. No explanation at the moment can be given for this phenomenon. Destruction of thrombin by "fibrinogen fraction" can, however, be excluded.

6. Effect of Fibrinogen Fraction on Prothrombin Consumption

Further tests showed that the one-stage prothrombin time of the incoagulable mixture of "fibrinogen fraction" with fresh blood was longer than 90 seconds. The prothrombin – factor VII content of the same mixture was determined after 1 hour incubation at 37° C. (prothrombin consumption) by the adsorption and elution technique. This method was used in order to eliminate the "fibrinogen fraction" from the system being tested. The prothrombin consumption in all the experiments was approximately the same as that of oxalated saline controls (Table IV). The spread of the values is due to the different volume ratios under which blood and the "fibrinogen fraction" were mixed. The high value of sample 1 is due to the fact that the eluate was

TABLE IV

PROTHROMBIN CONSUMPTION OF MIXTURE OF BLOOD OR NATIVE PLASMA WITH FIBRINOGEN FRACTION OR OXALATED SALINE

Samples of fibrinogen fraction	Prothrombin consumption (seconds)	
	Oxalated control	Fibrinogen fraction
1*	15.0	15.5
2*	11.6	12.6
3*	12.3	13.0
4*	10.0	9.0
5†	—	11.0
6*	11.0	11.0

*Fibrinogen fraction mixed with blood.
†1:1 mixture of fibrinogen fraction with native plasma. Hemolyzate was added.

mixed with adsorbed human plasma instead of rabbit plasma. Sample 5 consisted of a 1:1 mixture of "fibrinogen fraction" with native plasma. To the mixture hemolyzate was added to replace the centrifuged platelets (3, 6, 9). The results exclude destruction of prothrombin by the "fibrinogen fraction" and imply inhibition of its consumption.

7. Effect of Fibrinogen Fraction on Plasma Thromboplastin Generation

"Fibrinogen fraction" was added to native plasma, oxalated plasma, and serum in amounts slightly in excess of the minimum required to prevent coagulation of native plasma. Identical mixtures of oxalated saline with native plasma, saline with oxalated plasma, and saline with serum were used as controls. All the mixtures were left at room temperature for at least 1 hour and the thromboplastin generation test was then performed. One-tenth volume 0.1 *M* sodium oxalate was added to the tube containing native plasma in order to ensure proper adsorption on tricalcium phosphate. The already existing dilution with fibrinogen was taken into account when diluting with saline the plasma or serum to be tested. Table V and Fig. 5 show that in the thromboplastin generation test, only the native plasma had a reduced value. Normal results were obtained with all the other mixtures. This implies that when the "fibrinogen fraction" is added to plasma before the addition of sodium oxalate, the formation of thromboplastin in the thromboplastin generation test is reduced. On the contrary, when sodium oxalate is added to the plasma before the addition of the "fibrinogen fraction", the generation of thromboplastin in the test proceeds unimpaired. This points out that calcium is necessary for the action of the "fibrinogen fraction" on plasma

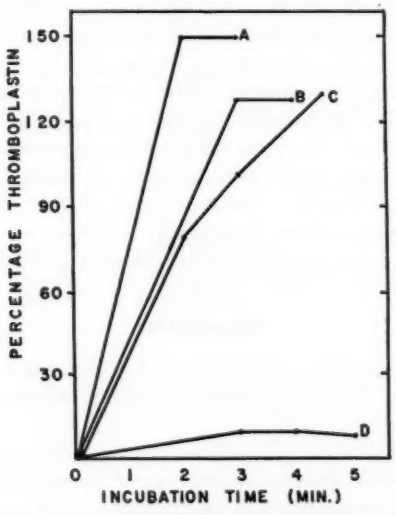

FIG. 5. Effect of "fibrinogen fraction" on thromboplastin generation. "Fibrinogen fraction" added to native plasma (D), oxalated plasma (C), and serum (A). Plain oxalated plasma was used as control (B).

TABLE V

EFFECT OF FIBRINOGEN FRACTION ON THROMBOPLASTIN GENERATION, FACTOR V, AND FACTOR VII

	Thromboplastin generation test (% of normal)*			Factor V (% of normal)*		Factor VII (% of normal)*	
	Native plasma	Oxalated plasma	Serum	Native plasma	Oxalated plasma	Native plasma	Oxalated plasma
Number of experiments	8	5	4	6	5	5	4
Control	101.0 (33)†	97.8 (26.8)	110.7 (33.6)	89.0 (12.6)	104.5 (11.5)	97.0 (6)	102.0 (35)
Fibrinogen fraction	13.6 (5.75)	95.0 (16.6)	122.0 (22.3)	23.7 (12.9)	94.5 (14)	114.5 (16.5)	103.0 (33.7)
P	<0.01	>0.5	>0.1	<0.01	>0.5	>0.1	>0.5

*Correction was made for the diluting effect of the added fibrinogen fraction, saline, or oxalated saline.
†Figures in parenthesis show standard deviation of the mean.

thromboplastin precursors. It is accepted that tricalcium-phosphate-adsorbed oxalated plasma furnishes antihemophilic globulin and factor V, while serum supplies plasma thromboplastin component (Christmas factor). This last factor remains unaffected, the thromboplastin generation test performed with the serum mixture being within normal limits (Table V). Consequently the results of the above experiments indicate that in the presence of the "fibrinogen fraction" and calcium a decrease in factor V or in antihemophilic globulin, or in both, must be assumed.

8. Effect of Fibrinogen Fraction on Factors V and VII

Factors V and VII were determined in 6:4 mixtures of "fibrinogen fraction" with native or oxalated plasma. To serve as controls, oxalated saline instead of "fibrinogen fraction" was added to native plasma and normal saline to oxalated plasma. All mixtures before being tested were kept at room temperature for 1 hour. One-tenth volume 0.1 *M* sodium oxalate was added to the native plasma – "fibrinogen fraction" mixture immediately before the determination of factor V, in order to facilitate the adsorptive action of tricalcium phosphate. The experiments (Table V) showed that only factor V of native plasma was reduced. The same factor in oxalated plasma remained unaffected. The concentration of factor VII was within normal limits in all mixtures. The difference between native plasma and its control is not statistically significant.

Discussion

The experiments described indicate that the "fibrinogen fraction" obtained between 25 and 50% saturation with ammonium sulphate has the following anticoagulant actions:

1. It inactivates factor V and reduces the formation of thromboplastin in the thromboplastin generation test, in the presence of calcium.
2. It inhibits the consumption of prothrombin.
3. It prevents the coagulation of fibrinogen by thrombin without destroying either of these two factors.

The reduced formation of thromboplastin in the thromboplastin generation test can be explained by a decrease in factor V and possibly in antihemophilic globulin, owing to a direct action of the "fibrinogen fraction". The fact that calcium is necessary suggests an alternative explanation; namely the possibility that the first phase of coagulation (formation of plasma thromboplastin) proceeds unimpaired in the presence of the "fibrinogen fraction". Thus, factor V and antihemophilic globulin are already consumed when the thromboplastin generation test is performed.

If the first of these hypotheses is correct, the impaired prothrombin consumption is due to a decrease of plasma thromboplastin. If the second is correct, we must assume that the "fibrinogen fraction" inhibits the action of normally formed thromboplastin on prothrombin. The prolonged one-stage prothrombin time may be partly explained by such an inhibiting action on the added rabbit brain thromboplastin.

The fact that the anticoagulant activity of incubated fibrinogen resides in the fraction which is precipitated between 25 and 50% saturation with ammonium sulphate suggests that this activity is linked with a large non-dialyzable molecule.

Fibrinolysin is excluded as the cause of the anticoagulant action because normal values of clottable fibrinogen were found in the mixtures of native plasma or blood with the "fibrinogen fraction".

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Addendum

The thrombin time prolonging effect of incubated fibrinogen has been recently reported by Stormorken, H. (Brit. J. Haematol. **3**, 299 (1957)).

THE ANTILIPAEMIC ACTIVITY OF LAMINARIN SULPHATE[1]

SAILEN MOOKERJEA[2] AND W. W. HAWKINS

Abstract

When rabbits were fed a diet containing 1% cholesterol the blood serum levels of neutral fat, phospholipid, free and esterified cholesterol, and β-lipoprotein were greatly increased. After the parenteral administration of laminarin sulphate or of heparin, all these were decreased, and the electrophoretic mobility of the β-lipoprotein was increased. Laminarin sulphate showed qualitatively the same effect as heparin on the hyperlipaemia. There was no clear relationship between the magnitude of dosage of either compound and the degree of its antilipaemic effect.

Introduction

The antilipaemic activity of heparin is reflected in effects upon the concentrations of glyceride (1–4), phospholipid (5, 6), and cholesterol (5–10), and there is a splitting off of lipid from β-lipoprotein (5, 11, 12), the plasma protein particularly involved in the transport of cholesterol (13). There is also evidence that heparin, presumably because of this property, inhibits the development of atherosclerosis in hypercholesterolaemic rabbits (6, 8, 14).

A number of compounds of relatively high molecular weight, most of them sulphated, have been shown to have antilipaemic activity. Some are chemically similar to heparin (15–23), and some are not (21, 24). Among them a preparation of laminarin sulphate with a low sulphate content (0.62 SO_4 groups per glucose unit) and a low anticoagulant activity (*ca.* 1/70 that of heparin) has been shown in human subjects to reduce hyperlipaemia and correspondingly to affect the β-lipoprotein (15).

We have used laminarin sulphate for which the laminarin was obtained from *Laminaria digitata* (25). It contains 1.7 sulphate groups per glucose unit (26), somewhat more than does heparin. Its molecular weight is about 10,000,* somewhat less than that of heparin. It has about one-third the anticoagulant activity of heparin, and appears to act like it principally as an antithrombin (20). It reduces alimentary lipaemia in dogs and rats (20).

The experiments described in this paper were designed to study in detail the antilipaemic activity of this compound, and to compare it with that of heparin. Rabbits were chosen as experimental animals because of the high levels of blood lipids, particularly of cholesterol, which can be induced in them by dietary means.

Procedure

Young rabbits of each sex, which weighed just under 2 kg. at the beginning of the experiments, were used. They were fed carrots and Purina rabbit

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*The molecular weight of laminarin is 4000–8000 (27), and this is approximately doubled by sulphation.

chow. For inducing hyperlipaemia the chow was treated to produce a mixture with 5% corn oil and 1% cholesterol. Each rabbit was fed about 100 g. of the chow preparation per day. On the blood serum of animals from both regimens, various lipid fractions were determined, and the electrophoretic pattern of the lipoproteins was obtained. For testing the antilipaemic effect of the anticoagulants, single intravenous injections of heparin or of laminarin sulphate were given at levels of 1 or 2 mg./kg. and 3 or 6 mg./kg. respectively, and blood was taken for examination of the serum ½ hour later. In other cases subcutaneous injections of 4 mg. of heparin per kg. or 12 mg. of laminarin sulphate per kg. were given twice daily for 2½–7 days before the serum was examined. These doses were chosen because of the relative anticoagulant effects of the two compounds (20), which of course may not be an important factor governing the antilipaemic activity.

Blood was taken from the animals by cardiac puncture. The levels of total lipid (28), total and free cholesterol (29), and phospholipid (30) were determined in the serum, and values for esterified cholesterol and neutral fat were obtained by difference. Electrophoresis of the proteins was carried out on paper by a procedure which followed essentially that described by Flynn and de Mayo (31). Ten strips of Munktell No. 20 filter paper 3 cm. × 42 cm. were run simultaneously in a system with barbiturate buffer of pH 8.6 and I 0.075. For the ordinary protein pattern 10γ of a 1:1 dilution of serum with buffer was used, and for the lipoprotein pattern 50γ of undiluted serum. Electrophoresis was carried out for 20 hours at 100 v. and 8–10 ma. Corresponding strips were stained separately with bromophenol blue for protein (32) and with Sudan black B for lipid (13). They were then scanned with a Photovolt densitometer, and from the readings so obtained graphical patterns of the distribution of protein and of lipid were made in the conventional forms.

Results

The results are shown in Tables I–III and Fig. 1. The tables present values obtained from the chemical analyses to show the partition of the serum lipids at different stages of the experiments. The figure shows examples of the electrophoretic picture of the serum lipoproteins of individual animals while they were on the normal and the high-cholesterol regimens, and before and after a single intravenous injection of anticoagulant.

Table I shows changes in the levels of serum lipids which occurred when the animals were on the diet with added fat and cholesterol. There was in general a progressive increase in all fractions, which in 6–7 weeks resulted in very high levels. The increases in cholesterol were particularly marked, and were of the same magnitude in both the free and the esterified fraction. There was an increase in the cholesterol/phospholipid ratio. The level of the phospholipid was the least affected, although increases in it were great. Accompanying the hyperlipaemia there was a marked increase in the β-lipoprotein in every case. This is illustrated in Fig. 1. An α-lipoprotein fraction was apparent on the electropherograms of sera from five out of nine animals, but it did not increase in hyperlipaemia.

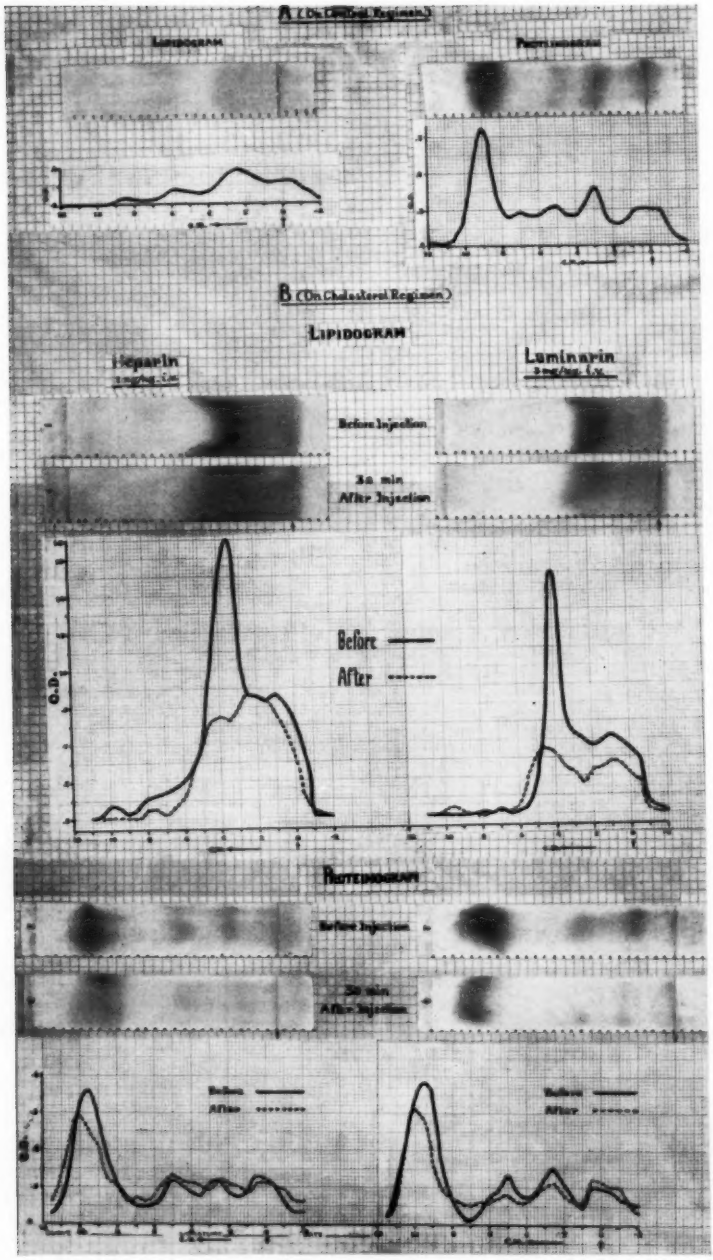

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TABLE I

LIPIDS IN THE SERUM OF NORMAL AND HYPERLIPAEMIC RABBITS

Days of cholesterol feeding	No. of animals	Ranges and averages. Concentrations in mg./100 ml.							
				Cholesterol					
		Total lipid	Neutral fat	Total	Esterified	Free	Ratio, tot./est.	Phospho-lipid	Ratio, chol./phos.
0 (normal)	9 (13 determinations)	220–449 *360*	66–268 *149*	39–58 *45*	18–32 *26*	15–26 *20*	1.6–1.9 *1.8*	90–187 *131*	0.3–0.4 *0.4*
7	5	1040–2000 *1326*	135–607 *276*	468–773 *598*	287–445 *348*	173–375 *250*	1.6–1.9 *1.7*	350–770 *474*	0.9–1.7 *1.3*
14	3	1740–3030 *2543*	358–433 *398*	895–1875 *1482*	580–1184 *921*	315–691 *561*	1.5–1.7 *1.6*	487–781 *663*	1.8–2.6 *2.2*
21	4	564–3150 *1325*	101–304 *185*	138–1946 *627*	89–1075 *357*	49–871 *270*	1.5–1.8 *1.7*	300–900 *513*	0.4–2.1 *0.9*
27–30	7	1108–3520 *2472*	125–1068 *655*	590–1775 *1151*	310–1125 *699*	280–650 *452*	1.6–1.9 *1.7*	393–875 *667*	1.3–2.6 *1.7*
42–47	5	3242–3890 *3474*	660–1017 *850*	1675–2030 *1905*	892–1285 *1087*	750–910 *818*	1.6–1.9 *1.8*	425–850 *722*	2.3–4.2 *2.8*
Average % increase over normal values in 42–47 days of cholesterol feeding		*865*	*470*	*4133*	*4081*	*3990*	*0*	*451*	*600*

TABLE II

THE EFFECT OF SINGLE INTRAVENOUS DOSES OF ANTICOAGULANT ON LIPIDS IN THE SERUM OF HYPERLIPAEMIC RABBITS

Days of cholesterol feeding	Dose	Sample, and diff. in level	Concentrations in mg./100 ml.							
			Total lipid	Neutral fat	Cholesterol				Phospholipid	Ratio, chol./phos.
					Total	Esterified	Free	Ratio, tot./est.		
27	Heparin, 1 mg./kg.	A (before inj.)	3520	872	1775	1125	650	1.6	873	2.0
		B (after inj.)	3164	780	1556	1088	468	1.4	828	1.9
		c (% change)	*−10*	*−11*	*−12*	*−3*	*−28*	*−13*	*−5*	*−5*
27	Lam.-SO_4, 3 mg./kg.	A	1570	259	756	448	308	1.7	555	1.4
		B	1100	297	425	288	137	1.5	378	1.1
		c	*−30*	*+13*	*−44*	*−36*	*−56*	*−12*	*−32*	*−21*
28	Heparin, 1 mg./kg.	A	1108	125	590	310	280	1.9	393	1.5
		B	960	74	538	288	250	1.9	348	1.5
		c	*−13*	*−41*	*−9*	*−7*	*−11*	*0*	*−11*	*0*
28	Lam.-SO_4, 3 mg./kg.	A	1944	610	756	388	368	1.9	578	1.3
		B	1720	505	660	340	320	1.9	555	1.2
		c	*−12*	*−17*	*−13*	*−12*	*−13*	*0*	*−4*	*−8*
42	Heparin, 2 mg./kg.	A	3380	755	1900	1150	750	1.7	725	2.6
		B	3160	980	1530	1016	514	1.5	650	2.4
		c	*−7*	*+23*	*−19*	*−12*	*−32*	*−12*	*−10*	*−8*
42	Lam.-SO_4, 6 mg./kg.	A	3242	1017	1800	987	813	1.8	425	4.2
		B	2460	1010	1050	610	440	1.7	400	2.6
		c	*−24*	*−1*	*−42*	*−38*	*−46*	*−6*	*−6*	*−38*
43	Lam.-SO_4, 6 mg./kg.	A	3890	848	2120	1285	835	1.6	922	2.3
		B	2600	775	1600	1025	575	1·6	625	2·6
		c	*−33*	*−9*	*−25*	*−20*	*−31*	*0*	*−32*	*+12*

TABLE III

THE EFFECT OF REPEATED SUBCUTANEOUS DOSES OF ANTICOAGULANT ON LIPIDS IN THE SERUM OF HYPERLIPAEMIC RABBITS

Days of cholesterol feeding	Dose	Sample, and diff. in level	Concentrations in mg./100 ml.							
					Cholesterol					
			Total lipid	Neutral fat	Total	Esterified	Free	Ratio, tot./est.	Phospholipid	Ratio, chol./phos.
30	Heparin, 4 mg./kg. twice daily for 4 days	A (before inj.)	3128	690	1765	1120	645	1.6	673	2.6
		B (after inj.)	2190	404	1301	898	503	1.4	485	2.7
		c (% change)	*−30*	*−41*	*−26*	*−20*	*−22*	*−13*	*−28*	*+4*
30	Lam.-SO_4, 12 mg./kg. twice daily for 3½ days	A	2892	960	1212	772	440	1.6	720	1.7
		B	1740	209	936	550	386	1.7	595	1.6
		c	*−40*	*−78*	*−23*	*−29*	*−12*	*+6*	*−17*	*−6*
47	Heparin, 4 mg./kg. twice daily for 7 days	A	3540	660	2030	1120	910	1.8	850	2.3
		B	2920	550	1650	1068	582	1.5	720	2.3
		c	*−18*	*−17*	*−19*	*−5*	*−36*	*−17*	*−15*	*0*
30	Lam.-SO_4 12 mg./kg. twice daily for 2½ days	A	3144	1068	1201	730	471	1.6	875	1.4
		B	1688	115	870	570	300	1.5	703	1.2
		c	*−46*	*−89*	*−28*	*−22*	*−36*	*−6*	*−20*	*−14*

Tables II and III show for individual animals the effect of injection of heparin and of laminarin sulphate upon the hyperlipaemia. All of the lipid fractions were affected, but quantitatively there were wide variations in response to the same dose. The usual pronounced effect on the level of cholesterol is notable. In some cases there were decreases in the ratios of total to esterified cholesterol and of cholesterol to phospholipid, but the occurrence and extent of these changes make their significance doubtful. The changes produced by laminarin sulphate in the serum levels of total lipid and total cholesterol were examined by Student's t test adapted for application to small samples (33). This was done using pooled data from Tables II and III on the various dosage levels and the two routes of administration. The test showed for total lipid a difference significant at a level between 2 and 5%, and for cholesterol at the 5% level.

The electropherograms showed in every case that after treatment with anticoagulant the β-lipoprotein fraction was decreased and its anodic mobility increased. This is illustrated in Fig. 1. On the strips which were stained with bromophenol blue there was always evidence of a slight increase in the mobility of the albumin fraction of the serum protein after the animals had been treated with anticoagulant.

Repeated subcutaneous injections generally had a more pronounced effect upon the serum lipids than the single intravenous injection of a smaller amount. This provided the only suggestion of a relationship between dosage and degree of activity. There was also a suggestion that laminarin sulphate was more effective than heparin in doses of the same anticoagulant potency.

There was a progressive gain in weight among all the animals throughout the experiments, and it was not affected by administration of the anticoagulants.

Discussion

Our experiments have shown that laminarin sulphate is an antilipaemic agent *in vivo*, that as such it affects the same serum lipid fractions as does heparin, and in the same way.

Any study of the properties which impart antilipaemic activity to a chemical compound is beset first of all with biological difficulties involving species and age of animal, and type and degree of lipaemia.

There is evidence that the effect of heparin upon blood cholesterol fractions is governed in part by the levels of those fractions themselves (7, 10, 11). This is important because it is known that there is a sex difference in the normal level of serum cholesterol in chickens (34) and humans (35), and in rabbits fed cholesterol (36). They are higher in females, although in humans not until later in life (35). Work with rabbits has indicated that the responsible factors could be sex hormones (36). It is also known that estrogen inhibits the production of lipaemia clearing activity by heparin in rats (37, 38).

FIG. 1. The effect of intravenous injection of heparin and of laminarin sulphate on the serum lipoproteins of rabbits.

Hormones of the adrenal and of the anterior pituitary glands also appear to be involved in the elaboration of lipaemia clearing activity under the influence of heparin (38, 39). In the rat, cortisone accelerates its production (38). In the rabbit, however, cortisone enhances the hyperlipaemic effect of cholesterol feeding (39); and heparin does not clear the lipaemia nor lower the cholesterolaemia (40) which may be induced in the rabbit by treatment with cortisone (39, 40).

The hyperlipaemia of some human diseases does not respond to heparin as does alimentary lipaemia, the differences being both qualitative and quantitative (6, 7, 41–43). Age is also a factor: it has been shown that heparin induces less clearing activity in people over 60 than in those 20–40 years of age (44).

It is thus apparent that the antilipaemic activity of a compound can vary depending upon a variety of circumstances. This point is made because of its importance both from the standpoint of testing and comparison, and in clinical considerations.

Among the sulphated polysaccharides with antilipaemic activity there is a wide variation in that property. In any one type of compound the molecular weight and the content of sulphate appear to be important. Anticoagulant property is apparently unimportant, and when it is present, doses too low to show it will still produce an antilipaemic effect (11, 17, 45, 46).

It has been shown with fractionated heparin that anticoagulant activity increases with the sulphate content (47). A relatively low sulphate content, however, is generally favorable to antilipaemic activity in a polysaccharide. Raising the sulphur content of sulphated alginic acid from 9.6 to 10.7% increases its anticoagulant activity, but a further increase to 19.7% abolishes its antilipaemic activity (17). Likewise sulphated cellulose is not antilipaemic when it contains 23.6% sulphur, but it is when the sulphur content is lower (17). The presence of some sulphur may be essential to the antilipaemic property of laminarin sulphate. There is evidence that antilipaemic activity is lost when the sulphate of this compound is lowered to between 0.37 and 0.62 sulphate groups per glucose unit (15). On the other hand, large intravenous doses of native glycogen and gum arabic have been reported to have antilipaemic effects (22).

A relatively low molecular weight appears to be favorable to antilipaemic activity in a sulphated polysaccharide. Depolymerized sulphated amylose has more activity than its parent compound (21), and heparin which has been degraded to the extent that it has lost practically all its anticoagulant activity maintains its antilipaemic activity (48). There is apparently, however, a limit to this, because sulphated carbohydrate compounds below a certain level of molecular weight have been shown to have no antilipaemic activity (17).

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METABOLIC EFFECTS OF ALPHA-TOCOPHERYL ACETATE

I. INFLUENCE OF ALPHA-TOCOPHERYL ACETATE ON SOME LIPIDS AND NITROGEN COMPOUNDS OF PLASMA IN HUMAN SUBJECTS[1]

DORIS E. GRAY AND SHIU-MAY LOH

Abstract

Significant increases in phospholipids, total cholesterol, and alpha-2 globulins and significant decreases in amino acids were found in the plasma of healthy medical students following daily ingestion of 100 mg. alpha-tocopheryl acetate (αTAc). The significance of these findings is discussed.

Introduction

Although numerous reports have been published on changes in the lipid and protein constituents of body tissues taken from vitamin E deficient animals, comparatively little information exists on the effect of administration of the vitamin to normal human subjects. Therefore it was decided to ascertain its effect, if any, on some lipids and nitrogen compounds in the plasma of healthy human subjects.

Experimental Procedure

Twelve healthy medical student volunteers were required to fast for 12 hours before each sample of blood was taken. During a preliminary control period four samples of blood were drawn from each subject, at intervals of 3 days, following which 100 mg. alpha-tocopheryl acetate (αTAc) was administered orally each morning for 10 days. Following this 10 day period, four samples of blood were obtained, after 12-hour fasts, and at approximately 3-day intervals. During this time blood levels of alpha-tocopherol were maintained by continuing the daily administration of 100 mg. αTAc.

Free and total cholesterol concentrations of plasma were estimated by the method of Schoenheimer and Sperry (1) and Sperry and Bland (2). Plasma phospholipid phosphorus was determined according to the method of Youngsbury and Youngsbury (3), and Fiske and Subbarow (4), and fatty acids by the method of Stoddard and Drury (5). Amino acid nitrogen was determined using the method of Frame, Russell, and Wilhelmi (6), and electrophoretic separation of the plasma proteins was carried out according to the method described by Liang (7). Where possible all estimations were performed in duplicate.

Results

Table I shows the plasma concentration of total cholesterol, free cholesterol, fatty acids, and phospholipids, in normal subjects, before and after ingestion of 100 mg. α TAc per day. Significant increases in the plasma levels of total and of free cholesterol and of phospholipid phosphorus followed the ingestion of αTAc. Fatty acid levels were not significantly influenced by the αTAc.

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Contribution from the Department of Physiology, University of Hong Kong, Hong Kong.

TABLE I

CONCENTRATION OF SOME LIPIDS IN HUMAN PLASMA, BEFORE AND FOLLOWING INGESTION OF ALPHA-TOCOPHERYL ACETATE

Plasma lipids	N	Control period Mean ± S.E.M.	Test period Mean ± S.E.M.	*P* value
Free cholesterol	12	54.9 ± 3.26	60.2 ± 3.15	<0.01>0.001
Total cholesterol	12	166.0 ± 9.64	178.8 ± 10.28	<0.01>0.001
Phospholipids	12	180.8 ± 10.96	204.4 ± 11.34	<0.01>0.001
Fatty acids	12	233.1 ± 12.18	245.2 ± 7.51	

NOTE: N is the number of values, each itself the mean from four samples. Estimations are expressed as mg. per 100 ml. plasma.

TABLE II

CONCENTRATION OF TOTAL AMINO ACIDS, IN HUMAN PLASMA, BEFORE AND FOLLOWING INGESTION OF ALPHA-TOCOPHERYL ACETATE

	N	Mean	±S.E.M.
Control period	6	6.6	±0.05
Test period	6	6.1	±0.19
P value	<0.05	>0.02	

NOTE: N is the number of values, each itself the mean from four samples. Estimations are expressed as mg. per 100 ml. plasma.

TABLE III

DISTRIBUTION OF PLASMA PROTEIN BY ELECTROPHORESIS

Component	N	Control ±S.E.M.	N	Test ±S.E.M.	*P* value
Albumin	24	53.7±0.98	24	52.8±1.49	
α-1-Globulin	24	4.1±0.27	24	4.1±0.45	
α-2-Globulin	24	8.3±0.31	24	9.3±0.45	<0.001
β-Globulin	24	12.6±0.53	24	12.5±0.45	
δ-Globulin	24	9.8±0.53	24	9.2±0.49	
γ-Globulin	24	11.5±1.02	24	12.1±1.39	

NOTE: Values are expressed as per cent total protein. N is the number of samples.

Table II shows the plasma concentration of amino acid nitrogen in the same subjects, before and after the ingestion of 100 mg. αTAc per day for 10 days. The amino acid nitrogen was significantly lowered following αTAc administration. Table III shows the electrophoretic distribution of plasma proteins before and after administration of the vitamin. A significant increase occurs only in the alpha-2 globulin fraction.

Discussion

Significant increases in total cholesterol, free cholesterol, and phospholipids in the plasma of healthy subjects were found after a daily intake of 100 mg. αTAc for 10 days. These increases in plasma lipids indicate clearly that the vitamin is implicated in the metabolism of fats. Similarly, a significant increase in the alpha-2 globulin component of plasma proteins, and a significant *decrease* in the plasma amino acids indicate that the vitamin is in some way connected with the metabolism of protein.

Increases in plasma cholesterol levels following vitamin E administration have been reported in both atherosclerotic rabbits (8) and atherosclerotic patients (9). Hove and Hove (10) observed that lipid depletion of arachodonate from the livers of rats fed with tri-*o*-cresyl phosphate can be prevented by administration of vitamin E, and further evidence that the liver is involved in the metabolism of vitamin E has been presented by Quaife (11), who demonstrated that the tissue most influenced by feeding alpha-tocopherol is liver.

The work of many investigators has suggested that a link may exist between vitamin E and protein metabolism. Dam (12) found that superimposing a protein deficiency on a vitamin E deficiency reduced the survival time in rats in comparison with vitamin E supplemented controls; Hove and Harris (13) demonstrated that the protein-efficiency ratio (total gain in body weight/protein consumed) was increased by the addition of alpha-tocopherol to a diet deficient in that vitamin; others have observed marked changes in certain tissues of experimental animals maintained on a diet deficient in vitamin E. Thus Olson (14) has shown that hepatic necrosis, which develops when weanling rats are fed diets devoid of vitamin E and low in sulphur amino acids, is prevented by administration of alpha-tocopherol and methionine, and that coenzyme A (CoA) content of liver varies inversely with the degree of hepatic necrosis. He has also shown (15) that acetyl CoA, a thioester of acetate and CoA, which is the active two-carbon fragment through which carbon and hydrogen of all oxidizable substrates of fat, carbohydrate, and protein must pass, can be increased by administration of vitamin E. That these changes involve sulphur metabolism has been demonstrated by Yang and Olson (16), who report that normal rats fed S^{35}-labelled cystine incorporate the cystine sulphur in CoA.

Milman, Silides, and Milhorat (17) found that less amino acid was released, *in vitro*, from the skeletal muscle of vitamin E deficient hamsters than from that of paired normal controls, and they have suggested that the reduction in protein breakdown may be secondary to a decrease in protein synthesis. In our laboratory, vitamin E administration was found to be attended by significantly lower concentrations of amino acid in plasma and by increased plasma protein concentration, suggesting that the increase in protein is made at the expense of the amino acids.

The electrophoretic patterns of plasma proteins show a significant increase in the alpha-2 globulin component following the ingestion of αTAc. Lewis,

Quaife, and Page (18) have reported that alpha-tocopherol is carried mainly in the alpha-2 globulin fraction of plasma proteins as lipoprotein complexes. Thus it seems likely that this fraction of protein is transported in plasma by the alpha-tocopherol as a lipoprotein complex. Olson (14) suggests that vitamin E protects the supply of sulphur amino acids in the body when they are scarce; Latner (19) has made similar statements based on clinical studies on patients with hepatic disorders. Olson and Dinning (20) have shown that hepatic CoA levels are lowest in necrosis, somewhat higher in animals on deficient diets but showing a little hepatic necrosis, and highest of all in those animals maintained on diets with alpha-tocopherol supplement. Thus one might expect an increase in synthesis of any compound which is dependent for its synthesis upon CoA, and it is suggested that, irrespective of substrate, the presence of alpha-tocopherol stimulates the synthetic process wherever CoA is required. According to Oliveira (21), vitamin E may be regarded as a component of a biological system and not merely as an antioxidant. Indeed it is generally acknowledged now that vitamin E has a specific and primary function, and a secondary non-specific action as a physiological antioxidant.

Nason and Lehman (22) have demonstrated in particulate preparations of rat skeletal muscle, the presence of a lipid cofactor functioning between cytochrome-*b* and cytochrome-*c* in the electron transport system, which can be substituted for specifically by the tocopherols, although in itself it contains no free tocopherol. These workers have made the tentative suggestion that the "co-factor or tocopherol acts as cementing material for maintaining cytochromes-*b* and *c* in correct spatial relation for optimal electron transfer". Thus the addition of vitamin E might result in the production of more energy, which would then be available for synthetic reactions. Whether vitamin E stimulates the synthesis of ATP directly, which in turn (provided the constituent molecules are available in sufficient quantity) could provide the energy for increased synthesis of CoA, remains to be established in the normal animal. Olson (15) has shown, however, that the amount of CoA in necrotic liver is inversely proportional to the degree of necrosis, and this would argue a dependence on vitamin E for CoA synthesis. It is, therefore, an interesting possibility that the action of vitamin E in increasing the concentration of lipids and protein of plasma is general, rather than a specific effect, dependent on the supply of CoA, which in turn could be dependent on the supply of ATP. Chernick and Schwarz (23) have concluded from their experiments with liver tissue from vitamin E deficient animals that the 'respiratory lesion' may involve a defect in ATP synthesis. In our laboratory, preliminary experiments with rat liver homogenates from animals maintained on large amounts of αTAc as a dietary supplement yield results which suggest that the liver cell from these animals is more active in the synthesis of fats and protein than is the cell of the normal liver. We are presently engaged in attempting to measure small amounts of ATP, as well as CoA in the liver cell, under varying conditions involving sulphur and vitamin E relationship.

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A HISTOCHEMICAL AND BIOCHEMICAL STUDY OF THE POLYSACCHARIDIC SUBSTANCES OF THE DEVELOPING NERVOUS SYSTEM OF THE RAT IN RELATION WITH THE APPEARANCE OF CHOLINESTERASE ACTIVITY[1]

MARC CREVIER[2]

Abstract

The PAS reaction and the cholinesterase reaction run parallel in the lower centers of the developing nervous system in the rat. Also, the two reactions appear progressively between the 10th and 15th day as the result of a chemical maturation of the neurons. Extraction of sections with methanol–chloroform does not alter the PAS reaction while acetylation inhibits it reversibly. These two facts suggest that 1,2-glycol groups are detected. Inhibition by hyaluronidase increases this evidence, despite the fact that diastase has a slight and irregular action. To elucidate the nature of the PAS reaction further, chemical fractionation of the brain substance has been attempted. (A) A fraction soluble in methanol–chloroform is metachromatic and PAS-positive *in vitro*. (B) A fraction soluble in a potassium chloride – potassium carbonate mixture is non-dialyzable, non-metachromatic, but PAS-positive. After acid hydrolysis it yields glucose but no hexosamine. (C) A fraction extracted from the residue by sodium hydroxide is non-dialyzable, non-metachromatic, but also PAS-positive. By chromatography and electrophoresis it is found to contain a non-sulphated mucoprotein yielding hexosamine in a high concentration following acid hydrolysis. The physiological significance of this mucoprotein is discussed.

Introduction

Among the polysaccharides presumably present in the central nervous system, Kerr (23) has been able to identify glycogen, and this finding has been confirmed by Brante (6), who has also been able to demonstrate that the greater part of hexosamine in the brain is not lipid bound and non-dialyzable. He concluded that there was a mucopolysaccharide, possibly linked to a protein. Moreover, in the calf, he isolated a strongly metachromatic substance, containing at least 30% hexosamine and moving rapidly toward the anode in paper electrophoresis. Finally he characterized a lipopolysaccharidic group of substances probably containing hexosamine and showing a positive metachromatic reaction.

The histochemical detection of the polysaccharides of the nervous tissue has been pursued almost exclusively by the periodic acid – Schiff procedure (PAS). In a survey of the distribution of the PAS-reactive carbohydrates in the rat, Leblond (26) has reported that some staining occurs in the cerebellum, especially in the molecular layer, as well as in the cerebrum. In both cases, a definite reaction took place in the gray matter while the white matter was negative. This author also observed that the cytoplasm of the neurons showed little or no reaction except in ganglionic cells and in the cells of the choroid plexus. Hess (19) studied the distribution of the PAS-positive

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substance in the central nervous system of rats and mice. He observed a red coloration between cells while the cell bodies, axons, myelin, dendrites, and neuroglia were usually negative except for the cells of the lower centers. On the other hand, in fresh frozen sections, Hess reported a positive PAS reaction in the myelin sheath, the reaction disappearing after extraction with lipid solvents. On this basis, he supposed that the reaction detected sphingomyelin and/or cerebrosides. Several other authors have reported that the motor and sensory neurons of mammals were PAS-positive and they stated that the reaction detected carbohydrate substances (35, 7, 34). More recently, the visualization of glycogen by the PAS procedure has been studied in the developing nervous system of *Amblystoma*. It has been shown that the reaction for glycogen first increases in intensity with the development of nervous tissues and then decreases (21).

The presence of cholinesterase activity in the neurons of the central nervous system is well established. Koelle has published a survey of the distribution of the enzyme in the rat brain (25). However, the appearance of cholinesterase activity in the developing nervous system has been the subject of few studies. Using the histochemical method of Koelle (24) Gerebtzoff (14) has reported that in the spinal cord and in the corpus striatum there is an early activity at birth, increasing sharply between the 13th and 15th day. On the other hand, Elkes and Todrick (11) have summarized the results of chemical determinations. They suggested that 8 and 22 days after birth was the critical period of observation in the development of acetylcholinesterase and cholinesterase in the rat.

The present work was undertaken in order to establish the developmental pattern of the PAS reaction in rats following birth. Moreover, it was of interest to study in parallel the development of cholinesterase activity during the early chemical differentiation of the neurons. Such studies are of interest since cholinesterase is somehow associated with a mucoprotein (3, 4, 30). Moreover, in addition to the histochemical investigations, biochemical experiments were conducted in order to establish more precisely the nature of the substances responsible for a PAS-positive test in the central nervous system.

The rat was chosen since it has been demonstrated that the newborn animal possesses an immature central nervous system (37). All the histochemical observations reported here will deal with the lower centers namely, spinal cord, medulla oblongata, pons, cerebellum, and olfactory bulb.

Material and Methods

Rats of the Sprague-Dawley strain ranging in age from 1 to 60 days were used. For the study of the PAS distribution, tissues were fixed in a mixture of one part neutral 40% formaldehyde and three parts ethanol for 20 hours or in 10% neutral formaldehyde in physiological saline, followed by the usual paraffin embedding and sectioning at 7μ. All through this work, we found no difference in the resulting PAS distribution or intensity following one or the

other of these two fixations. In performing the PAS reaction (20) the porcedure followed was that recommended by Gomori (16). The extraction of the lipidic substances that might give a PAS-positive test was carried out with a methanol–chloroform mixture, 80:20 at 40° for 20 hours. The prevention of the PAS reaction by the acetylation procedure and the reversal of this inhibition by potassium hydroxide was accomplished according to Gersh (15). Enzyme dissolution tests with hyaluronidase and diastase were conducted according to Lillie (27). Finally, for histological observation, sections were stained for 2 minutes with a 0.5% toluidine blue solution in 5% ethanol.

For enzymic studies, tissues were fixed for 18 hours in 4% formaldehyde in physiological saline at 4° and then frozen sections 15μ thick were prepared. The cholinesterase activity was visualized by the histochemical method of Crevier and Bélanger (9), using thiolacetic acid as substrate.

The biochemical investigations were conducted according to the following procedure: 10 rats, 60 days old, were killed by decapitation under light ether anesthesia. The brains were resected and most of the meninges were dissected out. The brains were then homogenized in absolute alcohol. The operation was performed within 2 minutes in order to avoid any post-mortem changes. The starting wet material amounted to 18 g.

The succeeding treatment was a modification of the Kerr method (22), which was thought to be milder and perhaps more efficient for polysaccharide extraction than the original.

(A) The fine pulp resulting from the homogenization was first extracted with several quantities of a mixture of 80:20 methanol–chloroform at 60° for 18 hours. The extraction was repeated until the extraction fluid appeared colorless after standing 1 hour in presence of the pulp. The extraction fluid was collected and evaporated to dryness. After emulsification in water, this material constituted the lipidic fraction.

(B) The remaining pulp was dried in the oven at 40° and twice extracted in 100 ml. of 30% potassium chloride in 1% potassium carbonate according to Einbinder and Schubert (10) for polysaccharide extraction. The yellowish extraction fluids were pooled and dialyzed for 24 hours against several changes of distilled water. The extract was then concentrated to 25 ml. under vacuum at 37° in a rotating evaporator.

(C) Finally, the resulting residue was further extracted with 20 ml. of sodium hydroxide 0.1 *N*, for 10 hours, dialyzed for 24 hours against several changes of distilled water, and concentrated to 10 ml.

Paper electrophoresis analyses of fractions B and C were conducted for 18 hours in veronal buffer (pH 8.6, ionic strength 0.1) at 100 v. and 55–135 ma. Papers were stained with amidoblack 10B (17) and mucicarmin (18). Densitometric curves of the strip diagrams were prepared according to Grassmann and Hannig (17). Ascending chromatography of the fractions B and C were run for 18–20 hours in a solvent consisting of 55% Na_2HPO_4 0.05 *M* and 45% isopropyl alcohol according to the method of Hamerman (18). The chromatograms were also stained with amidoblack 10B and mucicarmin.

Hydrolysis of the fraction B was accomplished in sealed tubes at 100° for 8 hours in presence of sulphuric acid 1.5 *N* and chromatography of the hydrolyzate was performed according to Partridge (32) in a mixture of butanol – acetic acid. The chromatograms were stained with ammoniacal silver nitrate (36). The fraction C was hydrolyzed during 6, 21, and 24 hours in sealed tubes at 100° in presence of hydrochloric acid 6 *N* and the hexosamine content of the hydrolyzate was determined by a modification of the Elson and Morgan method (31, 12). The *in vitro* test for metachromasia was performed by mixing 1 ml. of the fractions with 1 ml. of 0.05% toluidine blue in solution of 0.5% ethanol. The *in vitro* PAS test was accomplished according to McManus and Hoch-Ligeti (29).

Histochemical Results

The study of lower centers of the central nervous system included the spinal cord, the olfactory bulb, the cerebellum, the medulla oblongata, and the pons.

1. The Olfactory Bulb

The olfactory bulb has been studied in animals ranging from 1 day up to 30 days of age. Frontal, oblique, and longitudinal sections of the olfactory bulb have been prepared.

The PAS reaction becomes slightly positive in animals 10 days old in the glomeruli area and in the row of mitral cells (Fig. 5). The reaction increases in intensity at the 15th day (Fig. 7) and the tufted cells between the glomeruli and the mitral cells now appear stained. Then the reaction remains constant up to the 30th day.

The cholinesterase reaction follows exactly the same pattern of distribution but with a delay in the timing. At the 10th day, the reaction is barely visible at the level of the mitral cells (Fig. 6) but at the 15th day, it is very intense in these cells, in the tufted cells, as well as in the area of the glomeruli (Fig. 8). The reaction does not increase appreciably between the 15th and 30th day. In Fig. 9, a few enlarged mitral cells giving a positive cholinesterase reaction are shown illustrating their fancy shape. One cell may be seen with an intensely stained perikaryon while the nucleus is barely negative. In Fig. 10, a glomerulus is shown. The cholinesterase reaction is rather intense at this synaptic area (2) while in the capsule of small periglomerular cells, the same reaction becomes negative.

It must be said that the neurons of the olfactory bulb, as well as the synaptic area of the glomerulus, can be stained by toluidine blue as early as the first day following birth.

2. The Medulla Oblongata and the Pons

The medulla oblongata and the pons have also been studied. In this instance, no attempt has been made to identify the different nuclei but median sections of the brain have been cut at the same levels in order to compare identical areas.

The same observations were made in both cases, namely a very rapid spreading out of the PAS and cholinesterase reactions taking place between the 10th and 15th day following birth. At the 10th day, a very weak PAS and cholinesterase reaction can be observed in a few neurons as well as in the perivascular areas. At the 15th day, both reactions are definitely positive. Here again, the late chemical differentiation occurs in neurons which had already been stained by toluidine blue at birth.

3. The Cerebellum

The histological picture of the cerebellum of a newborn rat is different from what can be observed in a more mature animal. In sections stained with toluidine blue (Figs. 11 and 12) one can see that in a 1-day-old animal the Purkinje cells are not already present in a definite row and that they emerge progressively from the granular layer to take their characteristic position at the 10th day. This will be discussed later in regard to a related observation reported by Baffoni in an adult animal. At the 10th day, it can be observed that the PAS reaction is barely visible in the Purkinje cells while a diffuse reaction can be detected in the upper molecular layer (Fig. 13). At the same age, the cholinesterase reaction is negative in the Purkinje cells, but the same observation holds for the upper molecular layer (Fig. 14). At the 15th day, both the PAS and cholinesterase reactions become positive in the Purkinje cells while the same two reactions in the molecular layer are vanishing although the cholinesterase reaction in the granular layer is increasing in intensity (Figs. 15 and 16). At the 30th day, the PAS reaction has still increased slightly in the Purkinje cells while the cholinesterase reaction runs roughly parallel. It must be added, however, that the latter reaction becomes more intense in the dendrites of the Purkinje cells.

4. The Spinal Cord

The spinal cord gives a slight PAS-positive reaction as early as the 5th day following birth, this reaction being localized strictly in the motor cells of the anterior horn. At the 10th day, the PAS reaction is still mainly confined to the area of the anterior horn although a very weak reaction can be detected in the posterior horn. At that stage, the cholinesterase reaction is still negative. At the 15th day, both reactions run parallel, being more intense in each case for the motor cells of the anterior horn. One exception is the very strong cholinesterase reaction in the lateral horn (centroganglionic connectors) while a PAS reaction has never been visualized in this area (Figs. 17 and 18). At the 30th day, both reactions become still more intense in the neurons but also, at that time, a diffuse reaction takes place in the neuropile of the anterior horn.

5. Specificity of the PAS Reactions

The specificity of the PAS reaction has been questioned often (33, 28). Here we have studied the effect of acetylation and dissolution tests on this reaction. In every case, we have found that acetylation completely inhibits

the reaction which can be restored if acetylated tissues are submitted to mild potassium hydroxide hydrolysis. In the same way, Schiff's stain without prior periodic acid oxidation yields a negative result. The methanol–chloroform dissolution test does not change the reaction, while diastase (malt diastase) action might give a less intense reaction, although this dissolving action is irregular. The most striking result is obtained with hyaluronidase from beef testes. In this last instance, the reaction is inhibited especially at the level of the motor cells of the anterior horn.

Biochemical Results

1. Fraction A

The study of the fraction A was not extended beyond the test for metachromasia and the *in vitro* PAS test, as there was no methanol–chloroform extractable substance in tissue sections. Metachromasia was present and also a PAS positive test *in vitro* was detected (Table I).

TABLE I

BIOCHEMICAL CHARACTERISTICS OF THE THREE FRACTIONS EXTRACTED FROM RAT BRAIN

Fractions	Metachromasia	PAS *in vitro*	Hexosamine (as glucosamine)	Reducing sugar (as glucose)
A—methanol–chloroform	+	+		
B—KCl–K_2CO_3	0	+	0	+
C—residue extracted with NaOH	0	+	+	

2. Fraction B

The material which can be extracted by the potassium chloride – potassium carbonate mixture constitutes a complex and non-dialyzable fraction. Chromatography or electrophoresis analysis of the complex fraction showed that no spot could be stained either by amidoblack 10B or mucicarmin. Moreover, this fraction showed no metachromasia but a definite PAS-positive test *in vitro* (Table I). Following acid hydrolysis the fraction yielded only one reducing sugar which was thought to be glucose as a result of chromatographic evidence (Fig. 1).

3. Fraction C

The complex material which can be extracted from the residue by sodium hydroxide was first dialyzed. The test for metachromasia was negative, while the *in vitro* PAS test was positive (Table I). The results of an electrophoretic analysis are shown in Fig. 2. Stained with amidoblack 10B, the strip showed five peaks corresponding in position and relative intensity to those obtained with another tissue protein extract (1). When the strip was stained with

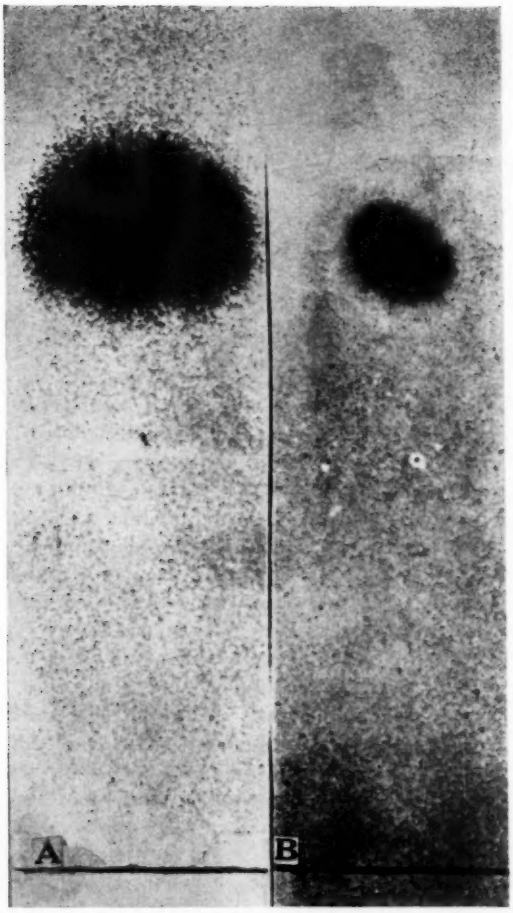


FIG. 1. A. Pilot chromatogram of the standard solution of glucose. B. Chromatogram of the hydrolyzate of fraction B. Volume applied: 25 µl. Solvent system: butanol–acetic acid.

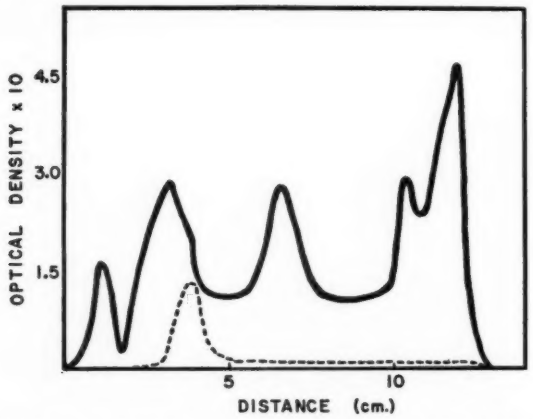


FIG. 2. Densitometric curves of paper strip diagrams following electrophoresis of fraction C in veronal buffer, pH 8.6. Continuous line: paper stained with amidoblack 10B. Broken line: paper stained with mucicarmin. Volume applied: 60 μl.

mucicarmin, which is rather selective for mucopolysaccharides, only one peak was present having a rather low mobility, while all other areas were unstained (Fig. 2).

In addition to this electrophoretic experiment, a chromatographic run was performed. For comparison purposes, three standard chromatograms were run with a 1.0% solution of egg albumin in phosphate buffer pH 8.6, with a 1.0% aqueous solution of chondroitin sulphate, and with a mixture of the two in equal proportions. Papers were stained first with amidoblack 10B and then overstained with mucicarmin. Results are shown in Fig. 3. Ovalbumin does not move in the solvent used except perhaps for a very slight tailing over the sharply circumscribed spot at the starting line (chromatogram C). Chondroitin sulphate alone moves along with the solvent to give an R_f of 0.45 and a rather well-defined spot (chromatogram B). This spot is not stained by amidoblack 10B and becomes visualized by mucicarmin. Finally, the mixture of chondroitin sulphate and albumin was resolved into two spots, the immobile albumin spot stained with amidoblack and the mobile chondroitin sulphate stained with mucicarmin (chromatogram A).

The chromatograms obtained with the extract from the residue (fraction C) showed the presence of a fast moving spot when stained with mucicarmin (Fig. 4, chromatogram A). When the chromatograms were stained with amidoblack 10B, two spots were identified, one at the starting point which was intense, and a second one at an R_f value similar to the one revealed with mucicarmin staining (Fig. 4, chromatogram B).

Because of the presence of a substance having the staining properties of mucopolysaccharides, an Elson and Morgan test for hexosamine was carried out on this fraction before and after acid hydrolysis. Before hydrolysis, the test was negative, but the hydrolytic process liberated a hexosamine in a rather high concentration (Table II).

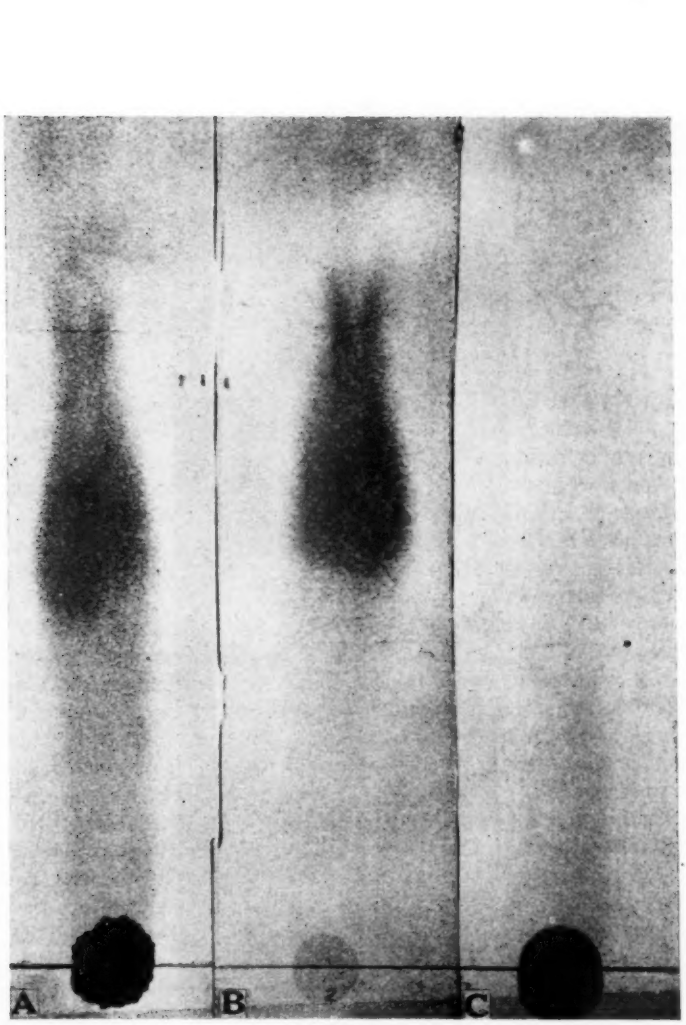


FIG. 3. A. Chromatogram of an equal mixture of ovalbumin and chondroitin sulphate. B. Chromatogram of a 1% solution of chondroitin sulphate alone. C. Chromatogram of a 1% solution of ovalbumin. Volume applied: 60 μl. Solvent system: isopropyl alcohol – Na_2HPO_4.

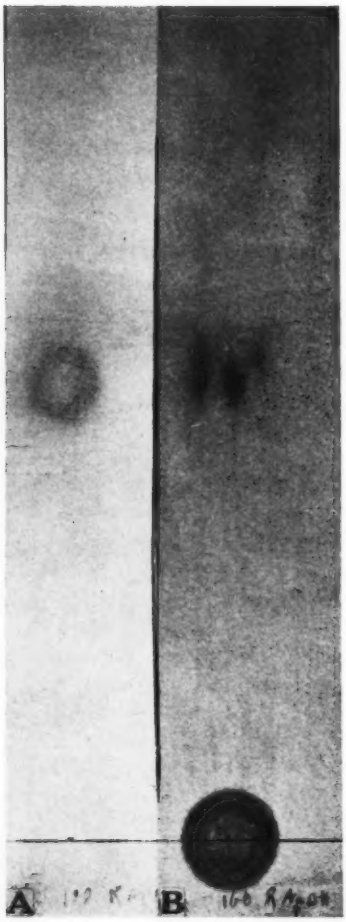


FIG. 4. A. Chromatogram of fraction C stained with mucicarmin. B. Chromatogram of the same fraction stained with amidoblack 10B. Volume applied: 60 μl. Solvent system: isopropyl alcohol – Na_2HPO_4.

TABLE II

HEXOSAMINE CONTENT OF THE SODIUM HYDROXIDE EXTRACT CALCULATED AS GLUCOSAMINE

Time of hydrolysis (hours)	μg. per ml.	Percentage of wet tissue
9	150	0.55
21	172	0.69
24	172	0.69

The greatest part of the hexosamine content is liberated in the first 9 hours of hydrolysis, a maximum value being reached at 21 hours and remaining constant after 24 hours.

Discussion

In all the histochemical experiments reported here, there is a striking parallelism between the PAS reaction and the cholinesterase reaction both in location and relative intensity. As far as the appearance of the reactions in relation to age is concerned, the cholinesterase reaction appears later, except perhaps in the case of the Purkinje cells, and the neurons of the pons and medulla oblongata. As far as the location and intensity are concerned, the following observations are made: In the olfactory bulb, at the 15th day, the two reactions can be definitely visualized in the row of mitral cells, in some tufted cells, in the area of glomeruli, and not elsewhere. The same applies to the neurons of the medulla oblongata and the pons, to the Purkinje cells of the cerebellum, and also to the spinal cord. In this last instance, the parallelism is more striking, since the motor cells of the anterior horn in both cases are the more active area. Moreover, at the 30th day, the neuropile and ground substance in this last area also becomes positive in each case.

In the experiments reported here, the appearance of the two reactions is a result of the maturation of the neurons around the 10th day following birth, and not from neurogenesis. Indeed, all the cellular elements to which reference has been made are already present at birth and can be stained by toluidine blue, with the exception of the Purkinje cells of the cerebellum. Baffoni (5) reported that the cells of Purkinje are in a state of continuous renewal from undifferentiated cells of the granular layer all through the animal's life. It has been demonstrated here that, at birth, these cells are not disposed in a characteristic row but arise progressively with time from the granular layer. At the 10th day, this peculiar disposition in a row is established. From then on, the chemical maturation is proceeding but with a slight delay in the time of appearance of the PAS reaction, which together with the cholinesterase reaction becomes definitely positive 15 days following birth. The specificity of the cholinesterase reaction has been discussed elsewhere (9). Both true and pseudocholinesterase are visualized by this reaction. However, Koelle (25) has proved that pseudocholinesterase is present in a very low concentration in the neurons of the central nervous system of the adult rat.

The nature of the PAS-positive substances has been the object of several discussions already reported in the literature (see for example Pearse(33), Lison (28)). Lipidic substances can give a positive test. In this present instance, prolonged extraction of the sections with methanol–chloroform mixture does not alter the intensity of the PAS reaction. It may well be concluded that lipidic substances do not take part in the histochemical reaction. However, the substances giving a positive metachromasia and a positive PAS test *in vitro* in the methanol–chloroform extract have been entirely dissolved by the organic solvents used in the histological procedure. There seems to be little doubt that the 1,2-glycol group can be specifically detected provided that special control experiments are performed. The fact that the acetylation procedure completely inhibits the PAS reaction makes it seem highly probable that the reaction is due to the presence of a 1,2-glycol group. Reversal of this inhibition following mild alkaline hydrolysis gives more strength to this hypothesis ruling out the possible role of acetyl phosphatides or inositol phosphatides. Indeed, this is an important point since Pearse (33) has reported that pyridine used as solvent in the acetylation procedure might dissolve these lipids giving a false impression of periodic acid oxidation blockade. The reversibility of the acetylation inhibition rules out this possibility.

FIG. 5. Frontal section of the olfactory bulb in a 10-day-old rat showing a weak PAS-positive reaction in the mitral cells and in the glomeruli. ×130

FIG. 6. Frontal section of the olfactory bulb in a 10-day-old rat showing a barely visible cholinesterase reaction in the mitral cells while the same reaction is still negative in the glomeruli. ×190

FIG. 7. Frontal section of the olfactory bulb in a 15-day-old rat. In comparison with Fig. 5, more intense PAS reaction in the glomeruli and in the mitral cells. Clearly visible reaction in the dispersed tufted cells between the glomeruli and the mitral cells. ×130

FIG. 8. Frontal section of the olfactory bulb in a 15-day-old rat. In comparison with Fig. 6, more intense cholinesterase reaction in the glomeruli and in the mitral cells. Clearly visible reaction in the dispersed tufted cells between the glomeruli and the mitral cells. ×190

FIG. 9. Few mitral cells stained by the cholinesterase reaction in a 30-day-old rat. The fancy shape of these cells can be observed together with a negative reaction in the nucleus. ×520

FIG. 10. A glomerulus stained by the cholinesterase reaction in a 30-day-old rat. The reaction is confined to the synaptic area while the capsule of periglomerular cells is negative in this respect. ×520

FIG. 11. Median section of the cerebellum in a 1-day-old rat stained with toluidine blue. The Purkinje cells are not yet disposed in a characteristic row. ×190

FIG. 12. Median section of the cerebellum in a 10-day-old rat stained with toluidine blue. The Purkinje cells are now disposed in a characteristic row after a progressive ascent from the granular layer. ×190

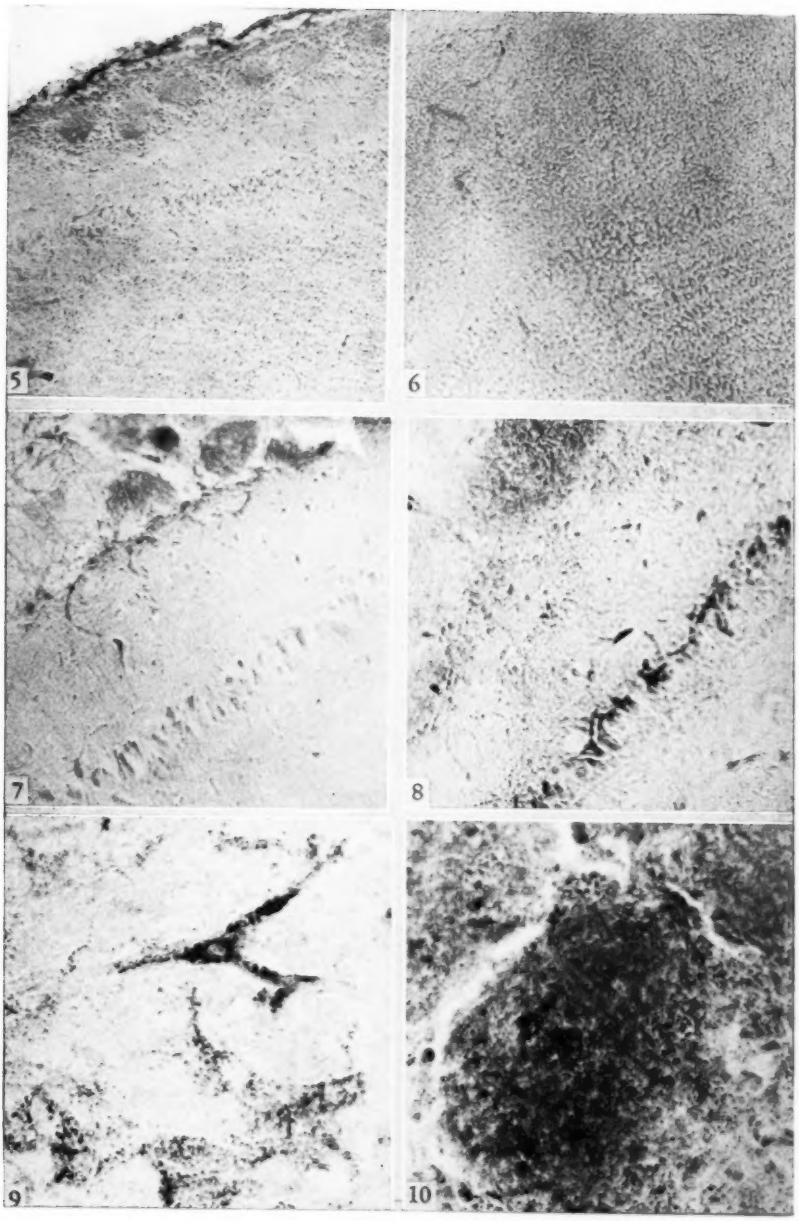
FIG. 13. Median section of the cerebellum in a 10-day-old rat showing a barely visible PAS reaction in the Purkinje cells. A diffuse reaction appears in the upper molecular layer. ×130

FIG. 14. Median section of the cerebellum in a 10-day-old rat. The cholinesterase reaction is almost negative except for a diffuse precipitate in the upper molecular layer. ×250

FIG. 15. Median section of the cerebellum in a 15-day-old rat. The Purkinje cells become positive and the reaction in the molecular layer tends to disappear. ×130

FIG. 16. Median section of the cerebellum in a 15-day-old rat. The cholinesterase reaction becomes positive in the Purkinje cells. ×250

PLATE I



Falling in the class of compounds having this 1,2-glycol group are glycogen and mucopolysaccharides. Glycogen is certainly present in the central nervous system, as we have been able to extract it with the potassium chloride – potassium carbonate mixture. Following acid hydrolysis of this fraction, only one reducing compound, glucose, could be characterized by paper chromatography. In view of the presence of glycogen, it is difficult to understand why the extended enzyme dissolution test of sections with malt diastase gives a barely visible result which, moreover, is irregular.

The mucopolysaccharides also containing the 1,2-glycol group might be involved in the PAS reaction. Hyaluronidase indeed fades the PAS reaction on the sections, indicating the presence of a mucopolysaccharide. On the other hand, extraction of the brain pulp with sodium hydroxide (fraction C) brings into solution a complex group of substances containing among them a compound which behaves like a mucopolysaccharide chromatographically and electrophoretically as revealed by the mucicarmin stain. Indeed standard chromatograms of chondroitin sulphate and albumin developed by the Hamerman procedure show that a mixture of the two components can be resolved in their two characteristic spots, each of them retaining their staining properties. When the sodium hydroxide extract from the residue is chromatographed by the same procedure, one spot can be visualized by mucicarmin, moving with the solvent, while two spots are resolved by amidoblack 10B, one appearing at the level of the mucicarmin stain and the second at the origin. From this experiment, it can be concluded that a mucopolysaccharide can be separated from the total protein fraction and that this mucopolysaccharide is combined with a protein, otherwise the amidoblack 10B stain would have been negative.

Electrophoretically, this mucoprotein complex has as low a mobility as that of γ-globulin indicating that it is certainly not a sulphomucoprotein (13, 8, 6). The lack of metachromasia and the presence of a positive PAS test *in vitro* with the same fraction also indicate this. Finally, the Elson and Morgan test for hexosamine indicates that this residue extract contains a fairly high amount of amino sugar calculated as glucosamine. The figures reported here are sensibly higher than those previously reported by Brante (6), calculated on the same basis. As this hexosamine is non-dialyzable and can be freed only after acid hydrolysis, there is no doubt that it is part of the complex substance present in fraction C which gives a positive test with mucicarmin after electrophoresis and chromatography.

All these findings would indicate that the PAS reaction revealed, together with glycogen, a mucoprotein in the central nervous system of the rat which is not a sulphomucoprotein. The presence of this mucoprotein might be of great physiological importance. Indeed, Bader and co-workers (3,4) have proved that a mucoprotein having a very high cholinesterase activity can be isolated from the serum. In the pancreas a similar mucoprotein has been reported as having the same enzymic activity (30). In the developing nervous system there is a close parallelism in location, relative intensity, and even

time of appearance between the PAS reaction detecting a mucoprotein and the cholinesterase reaction. This parallelism would indicate that the mucoprotein detected by the PAS reaction may be somehow associated with the cholinesterase.

Acknowledgments

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WEIGHTS AND COMPOSITION OF MUSCLES OF WARM- AND COLD-ACCLIMATED RATS[1]

O. Heroux

Abstract

After cold acclimation at 6° C. white rats have a smaller muscle mass than control animals of the same age kept at 30° C. Water and protein determinations in soleus and gastrocnemius muscles as well as in the whole muscular mass revealed that the reduction in the muscle mass after cold acclimation is a real tissue difference due to a reduced protein deposition and not only a water content difference. Apparently red and white fibers are equally affected in their growth by the prolonged cold exposure. Measurements of number and size of muscle fibers in soleus cross sections strongly suggest that protein deposition had been reduced in the same proportion in both muscle fibers and connective tissue.

Introduction

It has been reported recently (3) that after cold acclimation, white rats have a smaller body weight than controls of the same age mainly because they have a smaller muscle mass. Further experiments were done to ascertain whether the difference in muscle weight reflected a real reduction in muscle growth or a mere reduction in water content. A histological examination of the soleus muscle was also carried out to determine in what proportion muscle fiber and connective tissue were affected by the cold exposure.

Methods

Male Sprague–Dawley rats with a mean body weight of 204 g. (range: 145–242 g.) were used in two series of experiments. Each rat was kept in an individual cage, fed Master fox cubes and tap water ad libitum, and was weighed once a week.

First Series

Thirteen rats were randomly assigned to two groups. One group of seven rats was maintained at 30° C. and the other (six rats) at 6° C. for 8 weeks. After acclimation, the rats were weighed, killed with excess of ether, skinned, and eviscerated. After removal of all the free fat tissue and weighing of the carcass, the left soleus muscle was dissected and both the carcass and the soleus were desiccated in an oven at 98° C. until the weights were constant. After desiccation, the carcass was digested for 10 hours in a 10% KOH solution, then in a 30% KOH solution for 1 hour. The remaining bones were washed several times with this solvent. The supernatant solutions from each digestion were decanted, combined, and made up to a volume of 2 liters. After stirring, 1-ml. samples were transferred to micro-Kjeldahl flasks for nitrogen analysis (5). The whole soleus muscle was used for nitrogen analysis. The

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water content was obtained by the difference between the wet and dry weight of the muscles, the lipid content by the difference between the dry weight and the protein content (nitrogen × 6.25), considering the carbohydrate and ash content as negligible. According to data reported by Baker and Sellers (1), only 10 to 13% of the difference between dry weight and protein is actually glycogen.

In preliminary experiments, the wet and dry weights of bones remaining after the digestion of non-desiccated carcasses of 30° C. and 6° C. acclimated rats appeared to be essentially the same for both groups and quite similar to the skeleton weights reported by Donaldson (2) for Wistar rats of the same body weights. Assuming, on the basis of these preliminary results, 23 g. for the wet weight of the skeleton and a 34% content of skeletal water, it was possible to calculate the wet and dry muscle weight by subtracting the bone weight from the carcass weight.

Second Series

Twenty-one rats were randomly assigned to two groups. Group I (nine rats) was maintained at 30° C. for 4 weeks, group II (six rats) at 6° C. for the same length of time.

After acclimation, the rats were weighed and killed with excess of ether. The left leg muscles (gastrocnemius, plantaris, soleus) were dissected, weighed, and desiccated to constant weight in an oven at 98° C. The same methods as described above were used to obtain the water, protein, and lipid contents of these muscles.

The right soleus muscles were fixed in a 5% formalin solution, dehydrated in alcohol, cleared in toluol, and embedded in paraffin (Fisher Tissuemat, melting point 54–56° C.). Sections of 10 μ thickness were cut and stained with eosin and haematoxylin. On these sections, the number of fibers per unit area was counted in a sample area of 0.206 mm.^{2} at a magnification of 430×, and, with the aid of a camera lucida, the size of the fibers was measured in an area of 0.124 mm.^{2} at a magnification of 660×. These measurements were corrected for shrinkage caused by the fixation, dehydration, and staining processes, following the method described in a previous paper (4).

Results

Soleus muscle and leg muscles as well as the whole muscular mass appeared significantly smaller in cold-acclimated than in warm-acclimated rats (Tables I, II, III).

Wet and dry weights, water, protein, and lipid contents were all significantly smaller after cold acclimation.

The lower protein content in the muscles of the cold-acclimated rats accounted for 24% of the difference observed in the entire muscle mass, for 18% in the leg muscle, and 15% in the soleus.

The area of the soleus section was 32% smaller in the cold-acclimated than in the warm-acclimated animals (column A, Table IV). The muscle fibers themselves were also 32% smaller but with the variability in this measurement

the difference did not quite obtain the 5% level of statistical significance (column B). The number of fibers per unit area was greater in the cold acclimated group (column C) but the total number of fibers in the soleus section (fibers/mm.^{2} × total area − column A × C) was found to be essentially the same in both groups. Finally, the proportion of muscle fibers, namely the mm.^{2} muscle fibers per mm.^{2} soleus section, was essentially the same on the average in both groups (column B × C × 10^{-6}).

TABLE I

COMPOSITION OF SOLEUS MUSCLE OF WARM- AND COLD-ACCLIMATED RATS

Group	Wet weight, mg.	Dry weight, mg.	Water, mg.	Protein, mg.	Lipid, mg.
30° C. (10)	124.0±4.6†	30.8±1.1	93.2±3.6	25.2±0.8	5.60±0.8
6° C. (12)	77.3±4.2	20.6±1.0	56.8±3.3	18.1±0.8	2.48±0.4
Diff. 30° C.−6° C.	46.7±6.3**	10.2±1.5**	36.4±4.9**	7.1±1.4**	3.12±0.9**

NOTE: Number of animals is given in parenthesis.
**Statistically significant to the 1% level.
†Standard error.

TABLE II

COMPOSITION OF LEG MUSCLES OF WARM- AND COLD-ACCLIMATED RATS

Group	Wet weight, mg.	Dry weight, mg.	Water, mg.	Protein, mg.	Lipid, mg.
30° C. (9)	2229±70†	562±23	1678±57	508±6	54±9
6° C. (6)	1544±86	396±28	1148±70	380±7	16±11
Diff. 30° C.−6° C.	685±111**	166±35*	530±90**	12.8±9**	38±14*

NOTE: Number of animals is given in parenthesis.
*Statistically significant to the 5% level.
**Statistically significant to the 1% level.
†Standard error.

TABLE III

TOTAL MUSCLE MASS COMPOSITION

Groups	Body weight, g.	Muscle			
		Wet weight, g.	Water, g.	Protein, g.	Lipid, g.
30° C.	491±12† (7)	223.0±5.0	160.2±5.6	49.6±1.4	13.2±1.4
6° C.	384±13 (6)	166.5±5.4	121.5±2.8	40.8±1.5	4.4±1.5
Diff. 30° C.−6° C.	107±18**	56.5±7.4**	38.7±6.2**	8.8±2.0**	8.8±2.0**

NOTE: Number of animals is given in parenthesis.
**Statistically significant to the 1% level.
†Standard error.

TABLE IV

PROPORTION OF MUSCLE FIBERS IN A CROSS SECTION OF SOLEUS

Group	A Total area of soleus section (mm.²)	B Size of a fiber (μ^2)	C Number of fibers per mm.²	A×C Total number of fibers‡	B×C×10^{-6} Mm.² muscle fibers per mm.² soleus section‡
30° C. (6)	10.3±0.9	2166±246†	279±34	2745±159	0.568±0.04
6° C. (8)	7.0±0.4	1472±213	432±30	2963±137	0.609±0.04
Diff. 30° C.−6° C.	+3.3±1.0*	+694±325	−153±45**	−218±210	−0.04 ±0.05

NOTE: Number of animals is given in parenthesis.
*Statistically significant to the 5% level.
**Statistically significant to the 1% level.
†Standard error.
‡Average of products for individual animals.

Discussion

The lower protein content of the muscles after cold acclimation shows that the smaller muscle weight indicates a real reduction in growth of the tissue and not only a reduction in water content. The component of muscle tissue which was most severely affected was the fat, which was 55% smaller in the soleus, 70% smaller in the leg muscles, and 66% in the whole muscle mass. However, since fat comprises only 2 to 5% of the muscles, most of the difference in total wet weight of muscles is evidently due to water and protein changes. On a percentage basis, the difference in weight of the soleus (which is almost 100% red fibers) and of the leg muscles (which are approximately 60 to 75% white fibers) was the same. On the basis of these two samples, it can be assumed that red and white fibers are equally affected by cold acclimation.

The histological examination of the soleus muscle showed the same total number of fibers in warm- and cold-acclimated rats. It is therefore clear that degeneration of muscle fibers during cold acclimation, which was a remote possibility, did not occur.

The near identity of the proportion of muscle fibers per unit area in the two acclimation groups shows that the composition of the muscle remained the same after cold acclimation and therefore that growth of both muscle and connective fibers was equally reduced during acclimation.

Even if the error attached to these values was reduced by the measurement of many more fibers, it is evident that the slight difference between the proportional areas of muscle fibers in the groups (even if statistically significant) could not account for the 32% difference in the total surface area of the sections.

It is therefore concluded that after cold acclimation, white rats had a smaller muscle mass than controls of the same age because protein deposition had been reduced, apparently in the same proportion, in both muscles and connective fibers. Further work is being carried out in this laboratory to assess the endocrinological factors involved in these changes.

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SOME CONSTITUENTS OF THE URINE OF NORMAL AND SCHIZOPHRENIC INDIVIDUALS[1]

R. M. Acheson,[2] Rachel M. Paul, and R. V. Tomlinson

Abstract

The compounds present in urine pools from schizophrenic and normal male individuals and which give colors with diazotized sulphanilic acid, and which can be extracted with ethyl acetate or eluted with aqueous phenol from charcoal adsorbates, have been compared and a number identified. Of the over 80 compounds detected only three, which do not contribute markedly to the over-all coloring pattern, have appeared uniquely in the three urine preparations from schizophrenics. Urocanic, 2-acetamidobenzoic, and imidazole-4-acetic acid have been chromatographically detected in human urine.

Introduction

There is a considerable bulk of rather unspecific evidence suggesting that urine from schizophrenic individuals possesses constituents which are not present to the same concentration in urine from normal persons. Much of this evidence is of one general type. Reactive color-developing reagents have been added to urine of both normal and schizophrenic persons, and authors (10) have often claimed that the colors produced by the latter type of urine are the more intense. Most of the reagents used (diazotized aromatic amines, glyoxylic acid, and reagents which oxidize phenols, etc., to colored products) couple primarily with aromatic compounds which contain either benzene or pyrrole rings which may bear activating substituents. There are very many compounds of these types present in both "normal" and "schizophrenic" urine.

It has been reported from this Laboratory (9) in general confirmation of the work of Young *et al.* (14) that greater concentrations of compounds which yield colored derivatives with diazotized sulphanilic acid (DSA) occur in schizophrenic urine than in urine from other mental patients or from normal individuals. These compounds were detected by chromatography of the urine using *n*-butanol – acetic acid – water (4:1:1) on Whatman No. 1 paper followed by spraying with the DSA reagent; the greatest differences between the schizophrenic and other urines appeared to lie in the 0.8–0.9, 0.45–0.6, and 0.15–0.3 R_f regions. The chromatograms were "rated" according to the number and intensity of the spots observed; the schizophrenics had an average "rating" of 6.0 ± 2.5, and the normals of 2.8 ± 1.3. Later work confirmed the statistical difference (see Reference 10 for references) and two-dimensional chromatographic studies (8) indicated that compounds detectable with DSA were present in schizophrenic urine extracts, but not in normal urine extracts when such extracts were prepared under comparable conditions and comparable amounts were spotted on the chromatograms. These compounds

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[2]Visiting Research Associate from the Department of Biochemistry, Oxford University, England.

had R_f's of 0.86, 0.84, *0.70*, *0.68*, 0.58, 0.57, 0.55, 0.54, 0.53, 0.48, *0.40*, *0.40*, *0.35*, 0.24, 0.28, 0.24, 0.21, 0.19, and 0.08 in butanol–acetic acid–water. The figures in italics are those which fall outside the R_f ranges which initially showed the greatest difference between normal and schizophrenic urines. The results are not necessarily inconsistent as the original work was done with untreated urine. This contains many materials which are absent from the extracts and which will displace compounds from their usual positions on comparable chromatograms. Furthermore a weakly colored area appearing on a two-dimensional chromatogram may be quantitatively masked by grosser quantities of other materials appearing at the same place on one-dimensional chromatograms such as those used for the statistical studies. The quantitative data presented also showed that if the compounds in the schizophrenic urine extracts were present in quantities barely detectable by the DSA reagent at the levels examined, then concentrations of up to 40% of these would remain undetected in the normal specimens.

The object of this paper is to report the chromatographic identification of a number of compounds which give colors with diazotized sulphanilic acid.

Methods

Urine Samples

Pooled overnight samples [3] of urine were obtained from acute schizophrenics in the male insulin ward of the Provincial Mental Hospital, Essondale, B.C. There were approximately ten contributors, none of whom had been receiving any tranquilizers, to each pool. The individual urines were above normal[4] as regards their diazo-coupling "rating" (8, 9). Normal pooled male urine samples were obtained from hospital staff and from volunteers. All urine samples were acidified to pH 1 on collection and were stored at 0–5° C.

Extraction Procedure

The urine pools were successively treated as follows:

(1) Extraction with 3×0.5 volume of peroxide-free ether gave a pale yellow extract which was concentrated at room temperature to the point of crystallization.

(2) The aqueous layer was saturated with salt, extracted with 3×0.5 volumes of ethyl acetate and the extract concentrated as before.

(3) The aqueous layer was then shaken with 50 g. per liter of charcoal (Baker and Adamson, code 1551, lot M146) for three-quarters of an hour. The filtrate from the charcoal was colorless but still contained considerable quantities of the diazo-coupling compounds 104 and 105 (see Table II), and traces of others. The charcoal was collected, well stirred several times with distilled water, collected again, and well washed with water. This removed salt, etc., which otherwise interfered during chromatography. The charcoal was then stirred with successive amounts (1 liter per 100 g. charcoal for each

[3]Obtained through the courtesy of Dr. F. E. McNair, Dr. R. Halliday, Dr. J. E. Boulding, and Mr. Robert Baker.

[4]The "ratings" were kindly determined by Mr. Robert Baker.

elution) of crystalline phenol (7%) in water at room temperature until the extracts were colorless. Evaporation of the combined extracts at 40° *in vacuo* with the addition of distilled water until the distillate gave no reaction with ferric chloride, and subsequent concentration to the point of crystallization, gave a brown liquid which was directly applied to the chromatographic paper.

In order to see if the ethyl acetate extraction of large volumes of urine could be avoided, one batch of urine was divided into two parts. One portion was treated as before, while the second was directly treated with charcoal without salt saturation. The customary eluate from the charcoal was then extracted with ethyl acetate, and both this and the aqueous layer were compared chromatographically with the corresponding fractions obtained by the normal procedure. With the exception of several ethyl acetate-extractable steam-volatile phenolic acids, and 5-hydroxyindole-3-acetic acid, which disappeared, the corresponding fractions gave the same chromatographic patterns.

One box of charcoal was used for most of these experiments. The first pool of schizophrenic urine was treated with the charcoal deactivated with stearic acid (6%) according to Asatoor and Dalgliesh (4). As identical results were obtained with the untreated charcoal, the deactivation procedure was not used for the other pools. However, difficulties arose when a new box of charcoal with the same batch number from the same source was used. This sample adsorbed the DSA color-developing compounds very much more strongly than the first, and prolonged treatment with 7% aqueous phenol at room temperature neither eluted all the colored compounds nor all those which reacted with the sulphanilic acid reagent. A final treatment with phenol–water (25:75) at 60–70° for several hours was therefore given and this made the elution more effective.

Chromatographic Procedure

The three extracts were chromatographed on Whatman No. 1 paper (18½ in. ×22½ in.). Trial runs were made to determine the maximum quantity of each extract which could be applied to the paper without overloading, and this quantity was thenceforth used. The chromatograms were run in descending isopropanol–ammonia–water (8:1:1) in the long direction followed by fresh *n*-butanol – acetic acid – water (4:1:1) in the short direction and the ether and ethyl acetate extracts were also chromatographed by the method of Armstrong, Shaw, and Wall (3) in ascending isopropanol–water–ammonia (8:1:1) followed by the organic phase of a benzene – propionic acid – water (2:2:1) mixture. In the case of the last solvent equilibration of the paper is advised in Salt Lake City (3) but this proved unnecessary in the different atmospheric conditions of Vancouver. A number of authentic compounds were chromatographed in both homogeneous *n*-butanol – acetic acid – water (4:1:1) and the organic phase of the less convenient but often used 4:1:5 mixture. In most cases the R_f values obtained were the same within experimental error and in other instances the differences were small. The 4:1:1 mixture was therefore adopted here. It is of interest that the R_f values obtained with many of the authentic compounds are lower than those recorded

by Armstrong *et al.* in his solvents, but their relative values are virtually identical. The solvents used were of the best commercial grade. Many of the compounds, the properties of which are given in Table III, were obtained commercially while others were kindly donated.[5]

Spray Reagents

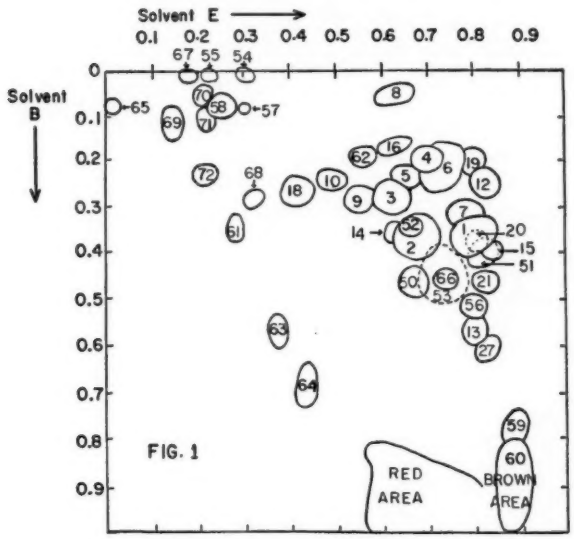
The modification by Armstrong *et al.* (2) of the Ehrlich spray (*p*-dimethylaminobenzaldehyde 2.0 g., in ethanol 140 ml. and concentrated hydrochloric acid 60 ml.) was used as the colors developed very rapidly, within a few minutes. Existing procedures (5) for spraying with diazotized *p*-nitroaniline gave such a deep red background due to decomposition to sodium *p*-nitrophenate that the colors of coupled azo derivatives could not be clearly seen. Virtually no background color was obtained with the following modification. The paper was sprayed lightly with (*a*) *p*-nitroaniline solution (5 ml., of 0.2% in *N* HCl) which had been mixed successively with hydrochloric acid (50 ml., of 0.1 *N*) and aqueous sodium nitrite (1 ml., of 5%) and allowed to stand in ice a few minutes after becoming colorless (it is important that the solutions should be below 5° when mixed in the sprayer, which should be kept cool in ice), and (*b*) immediately afterwards with ice-cold aqueous 20% potassium carbonate. The other sprays were prepared according to standard procedures and the ultraviolet (UV) source was a "Mineralight" short wave model SL 2537 with a short wave filter.

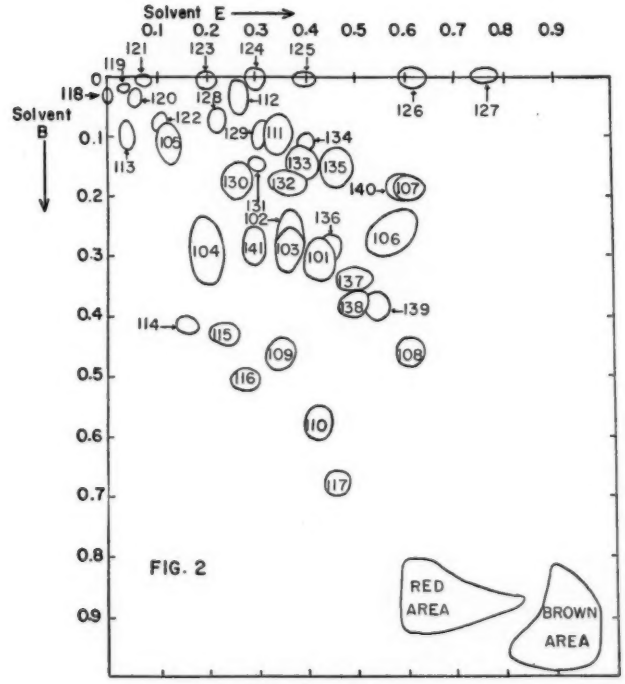
It is worth noting that the ninhydrin reacts with many compounds which are not aliphatic amino acids and that the Ehrlich reagent can also give purple colors with compounds which are not indoles but contain activated benzene rings. The cinnamic acids are resolved in some solvents (13) into their *cis* and *trans* isomers, usually one isomer being present in major quantities. Diazotized sulphanilic acid in general gives colored derivatives with compounds possessing activated aromatic nuclei through reaction of the diazo salt or excess nitrous acid. It can, however, give pale yellow colors with a number of compounds such as hippuric acid, phenylalanine, cinnamic acid, etc., under some circumstances. This may be due to a catalytic decomposition of the diazo salt yielding colored products, while in the case of phenylalanine it has been shown that the yellow color is due to several reactions yielding finally a diazoaminobenzene derivative (7).

Results and Discussion

The diagram of a typical chromatogram obtained from the ether or ethyl acetate extract of urines is shown in Fig. 1. After a small number of experiments it became clear that the ethyl acetate removed the last traces of compounds incompletely taken out by ether and a few additional materials. The preliminary extraction with ether was therefore discontinued. A list of compounds tentatively identified in the extract is given in Table I, along with other

[5]We thank Doctors C. E. Dalgliesh, J. Gardner, H. Tabour, A. Mehler, J. E. Boulding, and E. Schlittler of Ciba Pharmaceutical Products Inc., and Merck, Sharp, and Dohme Inc., The Upjohn Company, and the Sloan-Kettering Institute for these gifts.


Solvent E
0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9
Solvent B
0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9
67 55 54
70 65 68 57 69 71 8 62 16 4 6 19 5 12 72 68 10 18 9 3 7 14 32 2 1 20 15 51 61 50 66 53 21 56 63 13 27 64 59 60
RED AREA
BROWN AREA
FIG. 1


Solvent E
0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9
Solvent B
0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9
121 123 124 125 119 118 120 128 112 126 127 122 129 111 134 105 113 133 135 130 132 140 107 131 102 136 104 141 103 101 106 137 138 139 114 115 109 108 116 110 117
RED AREA
BROWN AREA
FIG. 2

FIGS. 1 AND 2

TABLE I

COMPOUNDS WHICH GIVE COLORS WITH DIAZOTIZED SULPHANILIC ACID AND SOME OTHERS DETECTED IN ETHER AND/OR ETHYL ACETATE EXTRACTS OF BOTH POOLED NORMAL AND SCHIZOPHRENIC URINES

No.	Compound or characterization	No. times detected in 3 pooled urine extracts: Normal	Schizophrenic
1	4-Hydroxyphenylacetic acid	3	3
2	3-Hydroxyphenylhydracrylic acid	3	3
3	3-Hydroxyhippuric acid	3	3
4	4-Hydroxyhippuric acid	3	3
5	5-Hydroxyindoleacetic acid	3	3
6	2-Hydroxyhippuric (salicyluric) acid	3	3
7	Homovanillic acid	3	3
8	Armstrong compound No. 8	3	3
9	4-Hydroxymandelic acid	3	3
10	4-Hydroxy-3-methoxy-mandelic acid (1)	3	3
11	3-Hydroxyphenylacetic acid	2	1
12	4-Hydroxybenzoic acid	3	3
13	2-Hydroxyphenylacetic acid	3	3
14	4-Hydroxyphenyllactic acid	3	3
15	3-Hydroxybenzoic acid	3	3
16	Feruloylglycine	3	3
18	Armstrong compound No. 18	2	3
19	Vanillic acid	1	3
20	Dihydroferulic acid	1	1
21	3-Hydroxyphenylpropionic acid	2	2
27	2-Hydroxybenzoic (salicylic) acid	2	2
33	4-Hydroxycinnamoylglycine	2	0
50	2,5-Dihydroxybenzoic (gentisic) acid	1	1
51	2-Aminobenzoic (anthranilic) acid	3	3
52	2-Aminohippuric acid	1	1
53	Hippuric acid	3	3
54	Altman red-purple	3	3
55	Altman red-purple	2	2
56	2-Acetamidobenzoic acid	2	3
57	DSA pink	2	2
58	DSA yellow-brown	2	3
59	DSA purple-brown	3	0
60	DSA brown	3	2
61	DSA yellow-brown	1	1
62	DSA pink-brown	2	1
63	DSA yellow	1	2
64	DSA yellow	0	3
65	DSA pale brown	1	0
66	N-Acetyltryptophan	2	1
67	DSA yellow-brown	2	1
68	DSA yellow	2	0
69	DSA brown	0	1
70	Blue-green phosphorescence	3	3
71	Blue-green phosphorescence	3	3
72	DSA gray	2	0

NOTE: The numbers used for most of the identified compounds are those of Armstrong *et al.* (3).

TABLE II

COMPOUNDS WHICH GIVE COLORS WITH DIAZOTIZED SULPHANILIC ACID AND DETECTED IN THE CHARCOAL ELUATES

Spot No.	Compound or characterization	No. times detected in 3 pooled charcoal urine extracts: Normal	Schizophrenic
101	Tryptophan (DSA pale yellow)	3	3
102	Imidazole-4-acetic acid (DSA red)	3	3
103	Tyrosine (DSA red)	3	3
104	DSA yellow	3	3
105	Histidine (DSA red)	2	3
106	DSA orange-yellow	3	3
107	Urocanic acid (DSA red-brown)	3	3
108	Phenylalanine (DSA very pale yellow)	2	2
109	DSA yellow	2	3
110	DSA yellow	2	3
111	DSA red-brown	3	3
112	DSA brown	3	3
113	DSA yellow	2	1
114	DSA yellow	2	0
115	DSA red-brown	2	2
116	DSA brown	2	1
117	DSA pale brown	1	0
118	DSA red	0	2
119	DSA red	1	2
120	DSA yellow	1	2
121	DSA yellow-brown	0	1
122	DSA yellow	1	1
123	DSA white	3	1
124	DSA yellow	3	1
125	DSA white	0	2
126	DSA pink →DSA gray	2	0
127	DSA white	0	2
128	DSA red	2	2
129	DSA red	2	0
130	DSA red	1	2
131	DSA blue	2	0
132	DSA red-brown	3	1
133	DSA white	0	3
134	DSA red	3	0
135	DSA pale yellow	2	1
136	DSA yellow	3	0
137	DSA orange	2	3
138	DSA white	0	3
139	DSA pale pink	2	0
140	DSA red-pink	2	0
141	DSA red	3	2

compounds which have not been identified but which give colors with diazotized sulphanilic acid. This list agrees very well with that of Armstrong *et al.* (3) with a few additions.

Comparatively few of the compounds eluted from the charcoal which are detected by the diazotized sulphanilic acid reagent (Table II) have been identified. It is very likely that a few of the compounds shown on Fig. 1 are not taken out efficiently by the solvent extraction and show up again on Fig. 2. For example, spots numbered 18 and 107 may be due to the same compound, the progress of which in the acidic solvent (Fig. 1) is held up by faster moving substances. The red and brown areas on both Figs. 1 and 2 are largely due to materials in the chromatographic paper, although additional materials giving rise to spot No. 60 (Fig. 1) are sometimes present. The "red area" may show discrete or very diffuse red spots which should not be confused with urinary constituents.

Tentative identification of compounds is given only when all the observable reactions and chromatographic properties of the compound agree with those of the authentic material (Table III) or with those of Armstrong, Shaw, and Wall (3). None of the compounds has actually been isolated, but the identifications have been confirmed by adding authentic material to the urine extracts and noting the increase in spot intensity at the appropriate place. The R_f values for specific compounds in the urine extracts are usually appreciably lower than those of the pure materials alone, because of the interference of the many other constituents. Of the compounds reported only 2-acetamidobenzoic, urocanic, and imidazole-4-acetic acids require comment as they have not previously been reported in human urine. Urocanic acid has been suspected as a metabolic product of histidine in man for some time but has hitherto only been noted as an occasional constituent of dog and rabbit urine (11). Imidazole-4-acetic acid is an enzymatic oxidation product of histamine (11); it is also formed from histidine in mussel tissue (12), and its presence to some extent in human urine is therefore not surprising. The identification of N^{α}-acetyltryptophan in normal human urine by Armstrong *et al.* (2), and the tentative identification of N^{α}-acetyl-kynurenine and its -3-hydroxy derivative in rat urine under special conditions (6) suggests that N-acetylation may lead to minor excretion products and the 2-acetamidobenzoic acid may be one of these.

Of the over 80 compounds which can be detected by the diazotized sulphanilic acid reagent only three have been consistently absent from the normal urine pools examined but present in all the schizophrenic urine extracts. Two of these give white colors, and the third a pale yellow, with the reagent and clearly contribute nothing significant to the "rating" observed by the procedure of McGeer *et al.* (8,9). One conclusion which can be drawn therefore is that the chromatographic "rating" can only reflect quantitative differences between schizophrenic and normal urines, and does not give any certain indication of a qualitative difference. There are also three unidentified compounds which have been detected in every normal pool and which were not observed

TABLE III

R_f'S AND REACTIONS OF STANDARD COMPOUNDS

Compound	Solvents A	B	C	D	E	F	UV	DSA	DNA	Alt.	Ehr.	Sil.	Ekm.	Nin.
Indoles														
N-Acetyltryptophan	58	57	54	–	83	75	Bl	pGy-Bn	pY	–	Pu[3]	Bn	–	pR-Bn
3-Dimethylaminoethyl-5-hydroxyindole (bufotenine)	94	84	18	–	49	51	Bl-Gn	R	Pi-Pu	–	Bl	Bk	–	Y-Bn
3-Dimethylaminomethylindole (gramine)	95	88	72	–	67	67	Bl	Y-Bn	C	–	Pi[9]	R-Br	–	C
5-Hydroxyindoleacetic acid	32	21	18	54	71	52	pR	R	Pi-R	–	Bl	Bk	–	Pi-Bn
5-Hydroxytryptamine creatinine sulphate (serotonin)	77	59	03	–	40	36	pPi	R	R	–	Bl[6]	Y-Bn	–	Pi-Bn
5-Hydroxytryptophan	35	16	00	02	17	40	N	R	Pi-Pu	–	Pu[10]	Bk	–	Pu
Indole	92	92	98	–	92	–	N	Y-Bn	Bn	–	R-Pu[8]	Gy-Bn	–	pY
Indole-3-acetic acid	61	50	84	–	88	68	Bl	pY-Bn	R-Bn	–	Pu[6]	N	–	pY
Indole-3-propionic acid	62	52	91	–	86	48	N	pY-Bn	pBn	–	Pu[3]	Bn	–	pBn
Indoxyl sulphate	88	70	00	–	32	63	Bl	pBn	Gy	–	O-Bn	N	–	N[6]
N-Methyltryptamine	95	85	–	–	69	61	Bl-Gn	W	pY	–	Bl	Bk	–	C[10]
Skatole	94	89	99	–	92	–	Bl	pY	Y	–	Pu[6]	Gy	–	pY
Tryptamine hydrochloride	86	74	55	–	64	50	N	pY	pBn	–	Pu[3]	Bk	–	N[6]
dl-Tryptophan	51	31	10	–	38	55	N	pY-Bn	N	–	Pu[10]	N	–	V
Iminazoles														
N-Acetylhistamine	89	75	19	–	52	87	N	R-Pi	Pi	N	N	N	N	N
Carnosine	27	14	01	–	10	92	N	Pi-R	Pi-Pu	–	N	N	N	N
Histamine dihydrochloride	77	51	02	–	21	84	N	Pi-R	R	–	pY	Bk	–	N[18]
L-Histidine hydrochloride	30	22	03	–	12	89	N	R	Pi-R	–	pY	Bn	–	V
Histidinol dihydrochloride	74	56	02	–	11, 21	90	N	Pi-R	R-Pu	–	pY	Bk	–	V
Iminazole-4-acetic acid	37	28	02	–	37	89	N	Pi-R	Pi	–	N	Bk	–	N
Urocanic acid	40, 70	29, 52	14	–	55	66	A	R-Bn	Pu	–	N	Bk	–	N
Hippuric acids														
2-Aminohippuric acid	54	38	43	–	72	75	Bl	pY	Y[13]	O	O-Y	N	R-Pu	N[6]
3-Aminohippuric acid	38	26	13	–	59	75	Bl	pY	C	O	Y	pY-Bn[12]	R-Pu	N
4-Aminohippuric acid	42	31	06	–	58	69	dBl[6]	pY	Y[16]	O	Y	Bn[12]	R-Pu	pBn
Hippuric acid	62	51	51	–	83	83	A	N[7]	N	O	N	N	–	N
2-Hydroxyhippuric (salicyluric) acid	27	22	39	–	83	–	Bl	Y	R	Y-O	N	N	–	N
4-Hydroxyhippuric acid	25	22	73	–	76	68	A	Y	Pi-R	O	pY	Bn	–	N
Nicotinuric acid	42	32	45	–	–	–	N	N	N	O-Pi	N	N	N	V
Phenaceturic acid	64	55	66	–	83	92	N	N	N	Y	N	N	–	N[21]
Picolinuric acid	55	46	70	–	79	89	A	N	N	O-Pi	N	N	N	N

Amino acids[a]														
2-Aminobenzoic (anthranilic) acid	55	51	91	–	87	75	Bl	Y	Y[13]	–	O-Y	Bn[12]	R-Pu	Y
3-Aminobenzoic acid	42	30	50	–	79	76	Bl	pY	Pi-Bn[18]	–	Y	Bn[12]	R-Pu	P[9]
4-Aminobenzoic acid	39	23	75	–	80	73	A	pY	Bn[13]	–	O-Y	Bn[12]	R-Pu	C[22]
5-Aminosalicylic acid	48	37	45	–	79	47	Bl	Br	Br	–	Y	Bn[12]	R-Pu	N
3-Hydroxyanthranilic acid	10	05	43	–	80	s	Bl-Gn	pBn	Gy-Bn	–	O	Bn	Bn	yBn
Kynurenic acid	65	50	19	–	51	32	Bl-Bn	N	N	–	N	N	–	N[21]
Kynurenine	41	29	14	–	30	71	G	N[7]	pBn	–	O-Br[11]	Gy-Bn	Pu[1]	Pu
Phenylalanine	54	48	32	–	52	90	N	pY	pM	–	pY	W	–	Pu
l-Tyrosine	51	30	03	–	29	83	N	R	Pu	–	pY	N	–	V
o-Tyrosine	61	37	08	–	40	77	N	O	Pu	–	N	N	–	V
Phenolic acids														
2,3-Dihydroxybenzoic acid	58	45	39	–	81	57	B	pPi	Gn-Gy	–	R-Bn	Bk	–	Gy-Pi[6]
2,4-Dihydroxybenzoic (*β*-resorcylic) acid	44	34	39	–	85	48	B	Bn	O-Bn[15]	–	N	Gy-Bn	–	N[11]
2,5-Dihydroxybenzoic (gentisic) acid	61	59	26	–	82	48	Bl	W	Y-Gn	–	N	Bk	–	N
2,6-Dihydroxybenzoic (*γ*-resorcylic) acid	89	79	06	–	54	47	A	Bn	O-Y[17]	–	pPu	pBn	–	N[11]
3,4-Dihydroxybenzoic (protocatechuic) acid	08	05	18	–	80	62	Bl	Pu	Gy[14]	–	N	Bk	–	N
3,5-Dihydroxybenzoic (*α*-resorcylic) acid	25	22	09	–	78	55	Bl	O-Br	O-Y	–	N	Br	–	N
3,4-Dihydroxycinnamic (caffeic) acid	11, 28	13, 20	22	–	77	23 31s	Bl	Pi-Bn	Gy	–	N	Bk	–	N[11]
2-Hydroxybenzoic (salicylic) acid	81	73	91	83	88	60	Bl	pY	R	–	N	N	–	N
3-Hydroxybenzoic acid	43	51	57	–	88	70	Bl	Y	R-Pu	–	N	N	–	N
4-Hydroxybenzoic acid	25	32	54	–	86	71	A	Y	R	–	N	N	–	N
4-Hydroxycinnamic acid	42, 55	29, 37	64	–	88	26	A	R	Gy-Bl	–	N	Bn	–	pBn
4-Hydroxy-3-methoxybenzoic (vanillic) acid	24	20	75	–	85	59	dBl[3]	O	Bl-Gy	–	N	Bk	–	N
4-Hydroxy-3-methoxycinnamic (ferulic) acid	27, 36	24, 34	81		85	33, 73	Bl	Pu-R	Bl	–	N[20]	Bk	–	N[11]
4-Hydroxy-3-methoxymandelic acid	38	41	12	–	62	84	A	Pi[4]	Pu	–	N	Bk	–	N
4-Hydroxy-3-methoxyphenylacetic (homovanillic) acid	39	32	72	–	84	77	Bl	R	Bl-Gy	–	N	Gy	–	N
4-Hydroxy-3-methoxyphenylpropionic (dihydroferulic) acid	51	43	86	–	84	70	N	Pi-M	Bl-Gy	–	N	Bn	–	N
2-Hydroxyphenylacetic acid	86	69	64	–	85	84	N	O	Pu	–	N	N	–	N
4-Hydroxyphenylacetic acid	43	41	46	54	85	81	N	R	Bl-Pu	–	N	N	–	N
4-Hydroxyphenyllactic acid	47	42	19	–	75	86	A	R-Pu	Pu	–	N	Bn	–	N
4-Hydroxyphenylproplonic acid	54	51	65	–	86	79	N	R	Pu	–	N	N	–	N
3,4,5-Trihydroxybenzoic (gallic) acid	02	03	02	–	56	47	A	C	Y-Bn	–	N	Bk	–	N
3,4,5-Trimethoxycinnamic acid	63	58	97	–	88	46	Bl	N	N	–	N	Bk	–	N
Xanthurenic acid	14	10	04	–	49	19	Bl	R	Pu	–	N	Bk	–	N

TABLE III—*Concluded*

Compound	Solvents						UV	DSA	DNA	Alt.	Ehr.	Sil.	Ekm.	Nin.
	A	B	C	D	E	F								
Miscellaneous														
2-Acetamidobenzoic acid	73	63	81	–	83	64	Bl	C	pC	–	O-Y[1]	W[1]	Pu[1]	N[2]
4-Acetoxy-3-methoxybenzoic (acetylvanillic) acid	28	22	95	–	88	80	A[2]	pO[1]	Pu	–	N	Bn	–	N
4-Acetoxy-3-methoxyphenylacetic (acetylhomovanillic) acid	55	37	95	–	89	89	Bl	pPi[3]	pPi[3]	–	N	Bk	–	N
Adrenaline	d	d	02	–	41	–	Y	Pi	Pi	–	pPi[9]	Bn	–	pBn
Cinnamic acid	65	58	98	–	89	s	A	N[7]	N	–	N	W	–	N[21]
3-Hydroxytyramine hydrochloride	d	–	05	–	43	73	N	Pi	Bn	–	N[10]	Bk	–	N[22]
Mandelic acid	65	61	62	–	82	–	N	N	N	–	N	pGy	–	N
Nicotinamide	91	73	80	–	66	75	A	N	N	N	N	W	N	N
Nicotinic acid	65	47	63	–	68	82	Bl	N	N	N	N	W	N	N
Noradrenaline hydrochloride	d	d	17	–	33	74	A	Pi	Bn	–	N[10]	Bk	–	M
Phenol	95	87	89	–	89	–	N	Y-O	Pi	–	N	N	–	N
Pyridoxal hydrochloride	58	40	24	–	79	86	A	pY	Pi	N	N	Bn	N	N[23]
Thiamine hydrochloride	d	d	ds	–	39	89	Bl	M	pPi	N	N	N	N	N
Tyramine	90	76	22	–	59	77	A	Pi	Pu	–	pY	Bk	–	N[20]

Solvents: (A) *i*PrOH:NH_3:H_2O, 8:1:1 by volume, ascending; (B) As (A), only descending; (C) C_6H_6:$EtCO_2H$:H_2O, 2:2:1 by volume, organic phase, ascending; (D) As (C), only descending; (E) *n*-BuOH:$MeCO_2H$:H_2O, 4:1:1 by volume, descending; (F) 20% KCl aq., descending.

A—absorbs; Bk—black; Bl—blue; Bn—brown; C—cream; d—decomposes; Gn—green; Gy—gray; M—mauve; N—no reaction observed; O—orange; p—pale; Pi—pink; Pu—purple; R—red; s—streaks; V—violet; W—white; Y—yellow.

Superscripts: [1]in 24 hr.; [2]Gn phosphorescence; [3]Bl-Gy in 24 hr.; [4]changes to O; [5]Bl phosphorescence; [6]Gy in 24 hr.; [7]pY occasionally noticed; [8]Bn-Pi in 24 hr.; [9]Bn in 24 hr.; [10]Gn in 24 hr.; [11]changes to Y on standing; [12]after spraying with 0.5% NaOH aq.; [13]Pi[12]; [14]Bn[12]; [15]Bl-Pu[12]; [16]O-R[12]; [17]Bl-Gy[12]; [18]pPu in 24 hr.; [19]Y in 24 hr.; [20]pBl in 24 hr.; [21]W in 24 hr.; [22]R-Bn in 24 hr.; [23]pR-Bn in 24 hr.

Reagents: DSA—diazotized sulphanilic acid; DNA—diazotized *p*-nitroaniline; Alt.—Altman reagent (*p*-dimethylaminobenzaldehyde in acetic anhydride); Ehr.—Ehrlich reagent; Sil.—ammoniacal silver nitrate; Ekm.—Ekman reagent (nitrous acid followed by *l*-ethylaminonaphthalene in dilute aqueous hydrochloric acid); Nin.—ninhydrin.

in any of the schizophrenic extracts. There is no evidence, as yet, which indicates a direct connexion between the three compounds detected unique to schizophrenic urine and the disease. The presence of these three compounds may be due to precursors in the hospital diet, or to other unknown factors. In view of the small number of pools examined, and the gross similarity of the DSA coupling compounds in schizophrenic and normal urine, it is clearly difficult to assess the significance of the few DSA positive compounds unique to each type of pool.

Acknowledgments

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METABOLIC AVAILABILITY OF ENERGY OF INGESTED ETHYL ALCOHOL[1]

R. J. G. Gillespie and C. C. Lucas

Abstract

Over 50 years ago Atwater and Benedict reported that the energy available from alcohol in metabolism is the same as that obtained in the bomb calorimeter. Mitchell suggested in 1935 that only about 75% is available for physiological purposes. The rate of growth of young rats is proportional to calorie intake (until this becomes optimal) when all other essential dietary factors are supplied in adequate amount. By taking advantage of this fact we have re-established that all the energy of ethanol found by bomb calorimetry is available in metabolism.

Introduction

Several hundred years ago physicians began to recognize that overindulgence in wines, beers, or distilled spirits leads to liver damage. Since alcohol is a common factor in all these beverages, clinicians came to attribute the fatty and cirrhotic lesions found in the livers of drunkards to a toxic effect of alcohol on the hepatic cells. This reasonable deduction remained unchallenged until recent years. Because alcohol is an energy-rich substance, the more of it one consumes, the less food one needs to maintain caloric balance. When it was recognized that the alcoholic patient combines a diminishing intake of protective foods with an excessive calorie intake, it seemed possible that the alcoholic might be suffering from an induced deficiency state rather than, or in addition to, a specific toxic effect of alcohol on hepatic cells.

Ashworth's conclusion in 1947 that alcohol exerts a toxic effect on the liver, even when the diet is adequate (2), was questioned by Best, Hartroft, Lucas, and Ridout (6). Equal intakes of extra calories as sucrose or alcohol caused identical damage to the liver in rats fed a marginal hypolipotropic diet and this damage was completely prevented by choline. The hepatic lesions (fatty and fibrotic changes) appeared to be accounted for by the imbalance of calories to vitamins (primarily choline). This conclusion has been questioned by Klatskin and his associates (15–18).

While Best *et al.* had confidence in the correctness of their conclusions, qualitatively, they were dissatisfied with two quantitative features of the study: (1) the possibility of occasional unsuspected loss of dilute alcohol from the water bottle (i.e. overestimation of alcohol consumption) and (2) what calorie value to assign to ethanol.

The energy value of ethyl alcohol (bomb calorimeter) is usually given as 7.11 Cal./g. There is some disagreement as to the availability of these calories in the body. In 1902 Atwater and Benedict (3) concluded that in man all

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Contribution from Banting and Best Department of Medical Research, University of Toronto, Canada. A preliminary report of this work (10) was given to the Division of Biological Chemistry at the 38th Annual Conference of the Chemical Institute of Canada at Quebec City, May 30, 1955, and a more complete presentation (11) was made at the 39th Conference, Montreal, May 28, 1956.

the energy is available in metabolism. Richter (25) found that alcohol could replace isodynamic quantities of food in maintaining energy balance in the rat and noted that its calories are used for growth and development in the same way as are those of carbohydrates. Ariyama (1) reported that the growth of rats was improved when alcohol was added to a high fat diet. These experiments seem to provide convincing evidence that alcohol resembles carbohydrates and fats, as a source of energy for the body. Most workers agree that alcohol can replace isocaloric amounts of carbohydrate and fat but Mitchell (20) contends that only about 75% of the energy of alcohol is available for physiological purposes. Morgan, Brinner, Plaa, and Stone (22) appear to be in agreement. Some controversy persists also as to whether the energy of alcohol is available for muscular activity as well as for heat production (21, 19).

The experiments to be described were undertaken to re-assess the availability of the energy of ethanol in rats maintained at normal room temperature and allowed the moderate activity possible in the conventional small individual rat cage.

It is a matter of experience that the rate of growth of young animals is proportional to the calorie intake provided that the other essential factors (water, minerals, vitamins, essential amino acids, etc.) are adequate and that the environmental temperature and exercise are similar. In spite of the small variability in activity of the animals and some variations in environmental temperature in our colony (21 ± 1°), the gains in weight of animals with equal calorie intakes displayed reasonably constant characteristics (Fig. 1). The slopes of these lines (i.e. the rate of gain in g./day) being typical of the calorie intake, it seemed feasible to make a moderately accurate estimate of the biological availability of the energy of ethyl alcohol by supplying part of the necessary calories in the form of dilute ethanol and noting the rate of gain.

A constant amount of a concentrated source of protein, minerals, and vitamins, completely adequate in nutrients but, at the level fed, deficient in calories was given to four groups of rats. Different increments of energy (provided as supplementary carbohydrate) were given to three groups of rats and the rate of gain on each calorie intake was noted. The rats of the fourth group were offered dilute alcohol (15% v/v) in place of drinking water. The rate of gain of this group, when compared with that of the others of known calorie intake, permitted an estimate to be made of the energy intake of these rats. The difference between the total calorie intake estimated in this way and the calories supplied by the solid food was attributed to energy supplied by alcohol.* With the grams of alcohol consumed and the calories contributed both known, the number of calories per gram may be readily estimated.

* The same procedure could, of course, be used to assess the energy available to the animal body from any other compound or natural foodstuff. Indeed, a recent abstract by Oser and Oser (24) proposes an identical method for estimating the biologically available energy of starches and gums.

Two separate experiments of this type have been conducted, in which the nature and amount of fat and of protein in the basal diets were different. Adequate minerals and vitamins were included in both mixtures. On the basis of the tentative estimated requirements proposed by Rose and his colleagues (27–29), both mixtures contained all the essential amino acids in more than adequate amounts. Supplementary calories were added in the first experiment as sucrose and in the second as glucose; the size of rat used was also varied. In spite of these differences the results were very similar: all the energy present in alcohol (as determined by the bomb calorimeter) appears to be available in the animal body for growth.

Experimental

Female rats (100–150 g., Wistar strain, raised in our own colony) were housed in individual all-metal cages with a false bottom of coarse wire screen. Accurately weighed amounts of fresh food were supplied daily; leftover and scatter were weighed the following morning. The rats were weighed daily (or sometimes on alternate days) at 10–11 a.m., about two hours after removal of food dishes. Fresh water was available at all times to all the rats except those given dilute alcohol (15% v/v) to drink.

The alcohol was dispensed from Gillespie drinking fountains (12) which collect all spillage and minimize losses by evaporation. Commercial 95% ethyl alcohol (10 liters) was redistilled (reflux ratio 4 : 1) from a 12-liter flask through a packed reflux column (1 meter long by 2.6 cm. inside diameter). When about 350 ml. had come over the *p*-hydroxydiphenyl test for aldehyde became negative. About 8 liters, distilled at a more rapid rate, was then collected for use in the experiment. By dilution of 150 ml. of this distillate to 1 liter with deionized tap water, a solution was obtained containing about 14.25% ethanol by volume: $d_{4^\circ}^{20^\circ}$ 0.97989 corresponds to 11.42% by weight or 11.19 g. of ethanol/100 ml.

Composition and Energy Content of the Diets

The ingredients of the basal food mixture fed in the two main experiments are shown in Table I. Different amounts of *sucrose* were added to a constant amount of the basal mixture in the first experiment, and of *glucose* in the second (Table II). To calculate the energy content of the basal dietary mixtures the conventional figures of 9.0, 4.0, and 4.0 Cal./g. were adopted for fat, carbohydrate, and protein, respectively. The compositions of the various foodstuffs were taken from tables in Jacobs' *Food and Food Products* (13). The energy values (Cal./mole) assigned to the supplements were those adopted by the International Union of Pure and Applied Chemistry, Lyons, France, 1923, viz. sucrose 1349.6, glucose 673.0, and ethanol 327.6, corresponding to 3.943, 3.733, and 7.111 Cal./g., respectively. Basal food Mixture A contained 35% protein and supplied 8.9 g. protein/100 Cal.; corresponding values for Mixture B were 33% protein and 8.7 g. protein/100 Cal.

TABLE I

COMPOSITION OF BASAL DIETARY MIXTURES

Component	Expt. 1 (Basal Mix A)				Expt. 2 (Basal Mix B)			
	Grams of component	Grams in mixture			Grams of component	Grams in mixture		
		Protein	Fat	Carbohydrate		Protein	Fat	Carbohydrate
Skim milk powder	20	7.2	0.2	10.4	15	5.4	0.15	7.8
Casein	5	4.6			4	3.7		
Alpha (soy) protein	5	4.8			4	3.8		
Fibrin	1	1.0						
Peanut meal (alc. ext'd)*					10	5.0		3.0
Whole wheat flour					10	1.2	0.2	7.3
Corn meal					10	0.9	0.4	7.4
Salt mixture (LP)*	3.0				1.0			
Vitamin–sugar powder*	1.0			0.9	1.0			0.9
Celluflour	4.8				0.8			
Sucrose	2			2.0				
Corn oil	8		8					
Lard					4		4.0	
Cod liver oil conc.*	0.020		0.02		0.010		0.01	
α-Tocopheryl acetate	0.010		0.01		0.010		0.01	
Choline chloride	0.20				0.20			
Totals	50.03	17.6	8.2	13.3	60.02	20.0	4.8	26.4
Calories		70	74	53		80	43	106
		Total Calories/50 g. = 197 Cal./g. = 3.9				Total Calories/60 g. = 229 Cal./g. = 3.8		

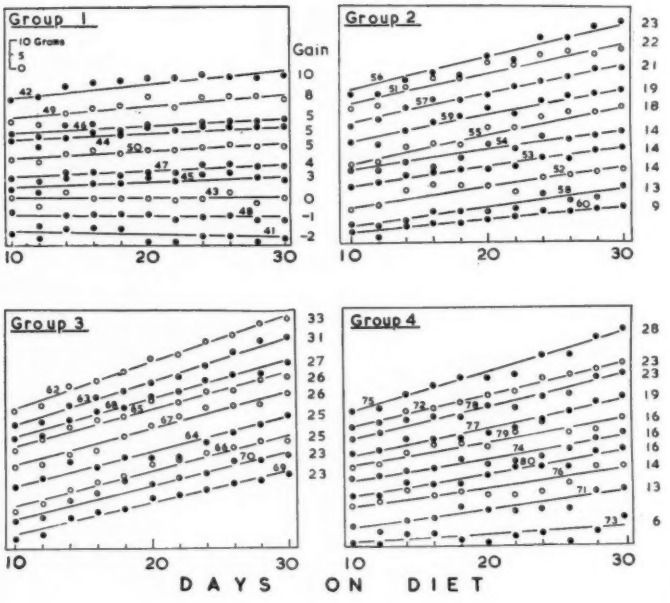
*Described in reference 26. Vitamin B_{12} (3 mg./kg.) was added to the vitamin mixture described.

TABLE II

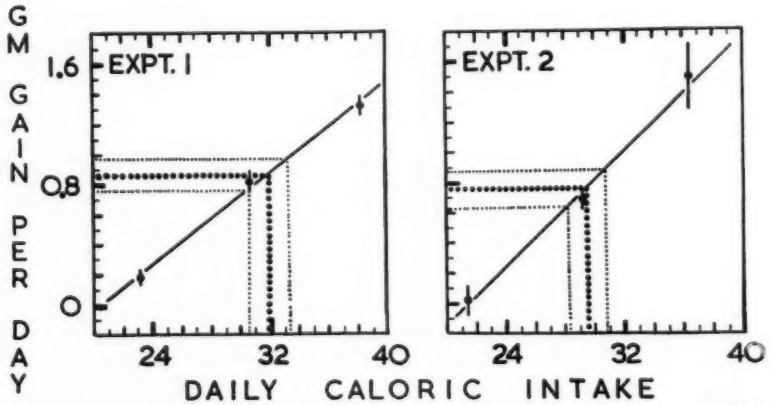
SUPPLEMENTED DIETS FED DAILY TO PROVIDE RATIONS OF INCREASING CALORIE CONTENT

	Expt. 1				Expt. 2			
	Diet A1	Diet A2	Diet A3	Diet A4	Diet B1	Diet B2	Diet B3	Diet B4
Basal mix, g.	5.0	5.0	5.0	5.0	6.0	6.0	6.0	5.0
Sucrose, g.	1.0	3.0	5.0	0	0	0	0	0
Glucose, g.	0	0	0	0	0	2.0	4.0	0
Food eaten, g.	5.9	7.8	9.7	4.9	5.6	7.7	9.6	4.7
Calories in food	23.2	30.7	38.2	19.3	21.4	29.2	36.4	17.9

NOTE: All rats received more than adequate amounts of protein, minerals, and vitamins so that calorie intake was the factor limiting growth.


Group 1
10 Grams
5
0
Gain
Group 2
Group 3
Group 4
10
20
30
DAYS ON DIET

1


GM GAIN PER DAY
1.6
0.8
0
EXPT. 1
EXPT. 2
24
32
40
DAILY CALORIC INTAKE

2

Protein Requirement of the Rats

Preliminary experiments established the dietary conditions under which the intake of calories rather than of protein limited the rate of growth. Seven groups of five male rats *(90–120 g.) were fed increasing amounts of Basal Mix B, viz. 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, and 8.0 g. respectively per day for 16 days. Sufficient sucrose was added to each portion to make the total food offered to each rat 10 g./day. All the rats (except those of Group 1) ate all the food; thus the energy intake was constant at about 39 Cal./rat/day. Rats in the first group lost weight throughout the test period; those given 2.0 g. of basal mix lost weight for 5 to 7 days and then began to gain slowly (0.9 g./day); 3.0 g. of basal mix permitted a distinctly better gain (2.4 g./day) and 4.0 g. still better (2.8 g./day). All three groups at the higher intakes gained at the same average rate (3.3 g./day).

The adequacy of the 5.0 g. portion was confirmed in another test at a considerably higher energy intake (about 60 Cal./day). The average rate of gain of five rats fed 5.0 g. Basal Mix B plus 11.0 g. glucose was found to be the same (3.5 g./day) as that of others fed 6.0 g. Basal B plus 10.0 g. glucose (average daily food intakes 14.6 g. and 14.3 g., respectively). Thus 5.0 g./day of this basal mix supplies liberal amounts of protein, minerals, and vitamins for a good rate of growth when calorie intake is adequate.

Estimation of the Physiologically Available Energy of Ethanol

Fig. 1 shows gains in weight of the individual rats of Expt. 1. The animals were given 10 days to adjust to the new environment and diets (Table II). The gain of each rat over the following 20 day period is shown at the right hand side of each set of curves. The total gain of the 10 rats of Group 1 was 37 g., averaging 0.19 g./rat/day. The total gain of Group 2 was 167 g. (average 0.83 g./rat/day). Group 3 (239 g. for nine rats) averaged 1.33 g./rat/day. The corresponding average daily intakes of energy were 23.5, 31.3, and 38.8 Calories, respectively. The 10 rats of Group 4 (total gain

*Male rats were used because they grow more rapidly than females, under optimal conditions, and thus have a somewhat greater requirement for dietary protein, minerals, vitamins, and energy. Requirements obtained with them would apply equally well to females but the reverse would not be true.

FIG. 1. Gains in weight of individual rats (Expt. 1). Small figures above the lines are the rat identification numbers. Figures opposite the right-hand ends of the lines denote the gains in weight (from slope of line) of the individual rats during the 20-day period. All rats were offered daily 5.0 g. of Basal Mix A; those of Group 1 were given 1.0 g. supplementary sucrose, Group 2 were given 3.0 g., and Group 3 were given 5.0 g. of sucrose daily. The rats of Group 4 were given no supplementary sucrose but were supplied with an aqueous solution (15% v/v) of ethanol as drinking fluid. The mean gains (with standard error) are: Group 1 3.7 ± 1.2; Group 2 16.7 ± 1.5; Group 3 26.6 ± 1.1; and Group 4 17.4 ± 2.0. (Corresponding data for Expt. 2 are given in Table IV.)

FIG. 2. Estimation of total daily calorie intake of rats drinking dilute ethanol. The average daily gains in weight of three groups of rats on different known calorie intakes are shown (○); lengths of the vertical lines through points give S.E. of mean; from the average gain in weight of the rats of the fourth group (drinking alcohol) their total calorie intake is estimated by following the heavy dotted line. The light dotted lines demarcate the limits ± 1 S.E. of the mean of the gains in weight of these rats.

174 g.) showed an average gain/rat/day of 0.87 g. This rate of gain corresponded to a total energy intake of 31.9 Cal./day (left hand graph in Fig. 2). The average food intake of these rats, 4.9 g., supplied 19.3 Cal./day, so that the energy derived from alcohol may be calculated by difference to be 12.6 Calories. The average daily intake of dilute alcohol by these rats (17.0 ml.) corresponded to 1.90 g. of ethanol, from which the metabolically available energy of ethanol can be calculated (see Table III) to be 6.6_3 Cal./g. The growth data of the second experiment, in which supplementary glucose supplied the increments of energy, are shown in Table IV and the calculation from these data of the physiologically available calories of ethanol, shown in the second column of Table III, gives 6.8_6 Cal./g.

TABLE III

CALCULATION OF ENERGY OF ETHANOL AVAILABLE PHYSIOLOGICALLY

	Expt. 1		Expt. 2	
Average gain of rats of Group 4 (g./day)	0.87		0.76	
Corresponding Calorie intake/day (from curve)		31.9		29.5
Average food intake (g./day)	4.9		4.7	
∴ Calories from solid food		19.3		17.9
∴ Calories from ethanol (by diff.)		12.6		11.6
Average daily intake dilute ethanol ($d_{4^\circ}^{20^\circ}$ 0.97989) ml.	17.0		15.1	
Average intake ethanol (g./day)	1.90		1.69	
Metabolically available energy/g. ethanol	6.6_3		6.8_6	

NOTE: Mean (6.7_5) is 95% of value by bomb calorimetry.

TABLE IV

GAINS IN WEIGHT OF INDIVIDUAL RATS OF EXPT. 2

Derived from slopes of best lines through weight data (cf. Fig. 1) from Days 9–30

	Group 1	Group 2	Group 3	Group 4
	+11	19	42	25
	+ 7	18	41	23
	+ 6	18	39	23
	+ 5	17	34	22
	+ 3	17	30	18
	− 1	17	28	16
	− 2	14	28	14
	− 7	10	28	10
	− 7	9	12	9
	−10	6	—	0
Total	5	145	282	160
Mean ± S.D.	0.5 ± 7.0	14.5 ± 4.6	31.3 ± 14.0	16.0 ± 7.9
Av./day ± S.E.	0.02 ± 0.10	0.69 ± 0.07	1.49 ± 0.22	0.76 ± 0.12

NOTE: Rats of Groups 1, 2, and 3 were fed Diets B1, B2, and B3, respectively (see Table II). The rats of Group 4 were offered 5.0 g. of Diet B4 daily and were given aqueous ethanol solution (15% v/v) to drink.

Discussion

The two estimates of the energy available to the body from ethanol agree remarkably well considering the many variables encountered in experiments of this type. Small amounts of alcohol may be lost from the breath, perspiration, and urine but there are relatively few accurate measurements directly applicable to our experimental situation. Bartlett and Barnet (4) state that the total lost by these routes varies from 2 to 10%. If one assumes a loss by rats of 5% of ingested ethanol, the mean value for the energy available in metabolism would be $6.7_5 \times 100/95 = 7.1_1$. This exact agreement with the value by bomb calorimetry can only be considered a phenomenal coincidence. The light dotted lines (standard error of mean of daily gains) in Fig. 2 show that there is a considerable degree of uncertainty as to what average rate of gain, and hence of total daily calorie intake, to assign to the rats of Group 4. In Expt. 1 the energy intake corresponding to the lower limit of rate of gain (0.77 g./day) was 30.7 Calories; using this value one calculates that the energy derived from alcohol was 11.4 Calories. This figure leads to the value 6.0_2 Cal./g. for ethanol or about 85% of the bomb value. The data for the upper limit (0.97 g./day, 33.2 Cal./day) correspond to a daily intake of 13.9 Cal. from 1.90 g. of alcohol or 7.3_2 Cal./g. for ethanol (103% of the bomb value). In Expt. 2, the variations in the primary data were somewhat greater yet the calculated energy content still fell within reasonable limits (86 and 105% of the bomb value, respectively).

Several other possible sources of error should be mentioned. The compositions of the various natural food products were mainly derived from food tables. In the few cases where analyses were made (e.g. the alcohol-extracted peanut meal) data obtained in our laboratory agreed with those in the literature. There is also a lack of unanimity as to the energy value to be assigned to the different dietary components (31). Small differences in these values will tend to shift the line in the graph slightly to the right or left on the horizontal scale of Fig. 2 since the position of the first point may not be known with absolute accuracy. The increments in energy due to the added sucrose (3.94 Cal./g.) or glucose (3.73 Cal./g.) are, however, definite values that lead to points of exactly known distances to the right of the first point. The estimate of the calories derived from ethanol utilizes the same starting point and hence any minor shift in the position of this point should not alter the conclusions since we are interested only in the improvements in growth associated with certain energy increments.

The above data confirm the finding of Atwater and Benedict, over 50 years ago, that all of the energy of ethanol is available in metabolism. For some years we have been curious how to account for Mitchell's claim that only 75% of the energy of alcohol is available in metabolism. Ethyl alcohol is readily soluble in water and does not require digestion. Ethanol appears to be absorbed almost instantaneously as it passes from the stomach to the intestine (9). Mitchell and Curzon (21) state that it is not only rapidly diffusible in aqueous solution, but that it appears to permeate animal membranes as

rapidly as water itself. Thus it would seem that any defect in the utilization of the energy of alcohol cannot be attributed to inefficiency in digestion, absorption, or diffusion of the compound in the tissue fluids or through the cell membranes. Loss by excretion of unoxidized alcohol is usually small; it varies from 2 to 10% (4), and is generally under 5% (30).

Bernhard (5) used isotopic ethanol, CD_3CD_2OD, and Bartlett and Barnet (4) used alcohol labelled with C^{14} to establish the generally accepted view that complete oxidation of ethyl alcohol occurs readily in the body, yielding the usual end products of animal metabolism, carbon dioxide and water. The oxidation to acetaldehyde is now well established in mammals as well as in microorganisms. The relative importance of alcohol dehydrogenase and of catalase in this process is still being debated. The ultimate conversion to acetic acid, known in lower forms since earliest times (viz. in the souring of wines and the manufacture of vinegar), was assumed to occur in the body of higher animals, although the intermediate metabolites are not fully known (see reviews by Carpenter (8), Newman (23), and Jacobsen (14)). If this does occur, the subsequent steps should follow the commonly accepted route of acetate metabolism (7) for it seems inconceivable that the cells of the body should be able to distinguish between acetyl units of different origin.

When the 'two carbon fragment' of the biochemist was recognized to be acetyl-coenzyme A and shown to be a common factor in the processes concerned with the mutual transformations of fat, carbohydrates, and protein, and in the liberation of energy from these basic foodstuffs via the citric acid cycle, there seemed to be no reason for the ineffective utilization of the energy of ethyl alcohol, either for heat production or muscular activity, as long as some source of pyruvate was available. Since Mitchell's animals were given food as well as alcohol, his findings appeared anomalous. Our curiosity about this prompted the investigation now reported.

After our manuscript describing these experiments had been written, a paper appeared by Morgan *et al.* (22) in which the conclusion was reached that only 75% of the calories of alcohol are utilized for growth in young rats given an

TABLE V

EFFECT OF ENERGY INTAKE ON GAIN IN WEIGHT/100 CAL.

	Expt. 1			Expt. 2		
	Total Cal./day	Weight gain, g./day	Gain/100 Cal.	Total Cal./day	Weight gain, g./day	Gain/100 Cal.
Group 1	23.2	0.19	0.82	21.4	0.02	0.09
2	30.7	0.83	2.71	29.2	0.69	2.23
3	38.2	1.33	3.49	36.4	1.49	4.09
4 (100%)*	31.9	0.87	2.73	29.5	0.76	2.58
4 (75%)*	28.8	0.87	3.02	26.6	0.76	2.76

*The total calorie intake per day has been calculated in two ways: in '4 (100%)' with the assumption that all the energy of alcohol is available for growth, and in '4 (75%)' with the assumption that only 75% is available. The findings for Groups 2 and 4 are in much better agreement when one assumes 100% availability.

adequate diet. However, the technique used for estimating the availability was not the most suitable, as the arrangement of *our* data in Table V reveals. Here it is seen that the gain/100 Cal. can vary over a wide range depending upon the calorie intake. It is known, of course, that it varies also with the size of the animal. Unless the animals are paired as to size and total energy intake per day, this kind of comparison is unlikely to permit an accurate estimate to be made of the degree of availability of the energy of alcohol. The method adopted in our present study, which seems more likely to give a reliable estimate of the metabolic availability, indicates that all the energy of ethanol released in the bomb calorimeter is available to the animal.

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RETICULO-ENDOTHELIAL SYSTEM STIMULATING COMPOUNDS AND THEIR MECHANISM OF ACTION[1]

B. GÖZSY AND L. KÁTÓ

Abstract

A series of compounds are described which exhibit therapeutic activity in experimental tuberculosis, acting exclusively on the host. The active compounds (citronellyl acetate, *d*-limonen, linalyl acetate) induce a local India ink and trypan blue accumulation and also stimulate the macrophages *in vitro* and *in vivo* to an increased phagocytic activity. Sera of the animals treated with therapeutically active compounds stimulate the macrophages of normal animals to an increased phagocytosis. A correlation between chemical structure of the reticulo-endothelial system stimulating compounds and the above effects is discussed.

Introduction

The curative effects of camomile flower have been known since Dioskorides and Paracelsus. Great physicians of the middle ages used it for the treatment of severe wounds and a variety of diseases. In recent times, it has been found that chamazulene, a component of the volatile oil of the camomile flower, is the active agent. The chemical structure of the chamazulene was revealed 2 years ago by Meisels and Weizmann (1) as 1,4-dimethyl-7-ethylazulene, but the substance has not yet been synthesized. A closely related compound, guajazulene (1,4-dimethyl-7-isopropylazulene) can be produced synthetically. Koch (2) and Janistyn (3) showed that guajazulene possesses the same pharmacological characteristics as the crude camomile extract. In our experiments (4) guajazulene showed a beneficial effect on experimental tuberculosis of the guinea pig. This beneficial effect was more clearly demonstrated when guajazulene was used simultaneously with subeffective doses of dihydrostreptomycin (DSM). Further experiments were carried out with a series of volatile oils and their components and several similarly acting compounds were found. Most of the active compounds had no action on tubercle bacilli *in vitro*. A few compounds were weakly bacteriostatic but the beneficial effect could not be attributed to the antibacterial action since bacteriostatic levels were not attained in the body following administration of therapeutically active doses. It was therefore concluded that the compounds were acting exclusively on the host. Experiments with guajazulene showed that this compound stimulated the fixed and mobile cells of the reticulo-endothelial system (RES). Guajazulene, if administered percutaneously in mice or rats, induces a local accumulation of intravenously injected India ink or trypan blue (5). Intramuscular administration of the compound leads to a systemic action which is reflected in an accelerated clearance of intravenously injected particulate matter from the blood stream (6). Macrophages from the peritoneal

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cavity of guinea pigs exhibit increased phagocytic activity *in vitro* in the presence of guajazulene (7). As similarly acting antituberculous compounds have been detected (8, 9) their action on the stimulation of the RES was studied. The results of these investigations are reported in the present study.

Materials and Methods

(*a*) *Screening of Volatile Oils and Their Components on Experimental Tuberculosis of the Guinea Pig*

Details of the method have been previously described (4). Each compound was tested on six to eight guinea pigs, infected subcutaneously with a 7-day-old culture of tubercle bacilli (H37Rv). Treatment was begun 21 days after infection and lasted for 8 weeks. The compounds were administered twice weekly and DSM was given daily.

Animals were autopsied on the day of death or as sacrificed. Visible macroscopic lesions were recorded in a schematic representation and results of treatment were calculated as described by Karlson and Feldman (10). Each organ was given a rating of 0 to 4, according to the amount of gross tuberculous disease. The index of tuberculosis is calculated in per cent and shown in the Tables I–III.

(*b*) *Stimulation of the Endothelial Cells of the Skin Capillaries*

Experiments were performed as previously described (5). With the help of this method information can be obtained about the local functioning of the cellular defence processes. This functioning is demonstrable by the India ink and trypan blue test. The intravenously injected dyes appear as black (India ink) or blue (trypan blue) spots at the site of the skin where compounds were administered. The intensity of the reactions depends upon the applied stimulus and upon its capacity to induce local phagocytosis. The most suitable animal for this investigation is the rat. One compound was tested on one side of the depilated abdominal skin of the rat and the skin on the other side served as control, where 10 μg. of histamine provokes in average a 1 cm. diameter spot, the intensity of which is registered as $++$. Each compound was tested on four rats, two with India ink and two with trypan blue. All volatile oils or their components were dissolved in 96% ethyl alcohol. If this solvent was rubbed into the skin no India ink or trypan blue storage was induced. All solutions contained 2% by weight of the tested compounds.

(*c*) *Stimulation of Phagocytic Activity of the Macrophages against Tubercle Bacilli (BCG)*

Details of the method have been previously described (7). Macrophages are produced in the peritoneal cavity of guinea pigs and harvested 6 days after intraperitoneal administration of glycogen. Ninety-six per cent of the cellular elements in the peritoneal exudate are macrophages at that time. The number of ingested BCG organisms were counted on stained slides or with contrast phase microscope. Per cent of macrophages were calculated ingesting 0, 1–2, 3–5, 6–10, and more than 10 tubercle bacilli.

The outcome of the tuberculous infection depends on the specific functioning of the macrophages, which are the only members of the cellular defence system that can ingest and digest the tubercle bacilli. Investigations on the stimulation of macrophages by guajazulene have been reported previously (7). The same type of experiment was now repeated with a series of volatile oil components. Guinea pigs were treated with the compounds for 5 weeks. Every fifth day, 10 mg. of the compound was injected intramuscularly in 0.5 ml. of olive oil. Control animals received the solvent. At the fifth week, 0.1 mg. of glycogen in 10 ml. of saline solution was injected intraperitoneally and macrophages were gathered 6 days later. Phagocytic activity of macrophages, as well as stimulating action of serum of the treated animals, was studied against tubercle bacilli (BCG).

Experimental Results

In Table I, antituberculous activity (TB index) of compounds is compared with the stimulation of macrophages and induced India ink and trypan blue storage. Compounds which showed therapeutic activity also stimulated the macrophages and gave positive skin reactions (guajazulene, citronellyl acetate, vetivert acetate, *d*-limonen, linalyl acetate). Compounds having no therapeutic effect or weak therapeutic action had no stimulating effect on the fixed and mobile cells of the RES.

TABLE I

ANTITUBERCULOUS ACTIVITY COMPARED WITH THE EFFECT OF STIMULATING PHAGOCYTIC ACTIVITY OF MACROPHAGES AS WELL AS INDIA INK AND TRYPAN BLUE STORAGE IN THE SKIN OF RATS

Macrophages from treated guinea pigs	TB index	Number of bacilli per monocyte: 0	1–2	3–5	6–10	>10 (% macrophages)	India ink	Trypan blue
Control	76.5	32	19	24	14	11		
Guajazulene	18.7	14	17	19	30	20	+++	+++
Citronellol	75.1	32	24	29	9	6	±	±
Citronellyl acetate	12.5	12	12	13	33	30	+++	+++
Vetiverol	42.1	36	19	24	11	10	+	+
Vetivert acetate	19.3	21	29	33	8	9	+++	+++
Linalool	20.6	34	22	26	11	7	+	++
Linalyl acetate	3.1	9	8	20	33	30	++++	++++
p-Cymen		37	23	18	12	10	−	−
d-Limonen	13.8	7	13	13	38	29	+++	+++

NOTE: TB index was calculated at autopsy after 8 weeks treatment with the compounds plus subeffective doses of DSM. Each group represents the average TB index in six or eight guinea pigs infected with virulent tubercle bacilli.

Macrophages were obtained from the peritoneal cavity of guinea pigs after 8 weeks treatment with the compound. One hundred monocytes were counted classified as ingesting 0, 1–2, 3–5, 6–10, and more than 10 tubercle bacilli 1 hour after incubation.

India ink and trypan blue reactions were obtained in the skin of rats 1 hour after percutaneous administration of the compounds. Dyes were injected intravenously and readings were made 1 hour later on the depilated and removed skin at the site where compounds were administered.

TABLE II

ANTITUBERCULOUS ACTIVITY COMPARED WITH THE EFFECT OF STIMULATING PHAGOCYTIC ACTIVITY OF MACROPHAGES AS WELL AS INDIA INK AND TRYPAN BLUE STORAGE IN THE SKIN OF RATS

Origin of opsonin	TB index	Number of bacilli per monocyte					India ink	Trypan blue
		0	1–2	3–5	6–10	>10		
		% macrophages						
Normal guinea pig	76.5	32	19	24	14	11		
Guajazulene treated guinea pig	18.7	10	15	16	36	24	+++	+++
Citronellol treated guinea pig	75.1	23	44	17	12	4	±	±
Citronellyl acetate treated g.p.	12.5	22	16	30	16	16	+++	+++
Vetiverol treated guinea pig	42.1	26	22	32	8	12	+	+
Vetivert acetate treated g.p.	19.3	18	15	24	27	16	+++	+++
Linalool treated guinea pig	20.6	24	25	28	16	7	+	+
Linalyl acetate treated g.p.	3.1	14	10	12	36	28	++++	++++
p-Cymen treated guinea pig		30	25	21	12	12	−	−
d-Limonen treated guinea pig	13.8	13	8	10	37	32	+++	+++

NOTE: Legend as for Table I, except that sera of the guinea pigs treated during 5 weeks with the tested compounds were added to macrophages of untreated guinea pigs in the experiment for screening the phagocytosis-stimulating effect of compounds.

In another series of experiments, sera from treated animals were added to macrophages from untreated animals. Normal serum was added to normal macrophages in the control tests. Results are shown in Table II and are comparable with those of Table I. Macrophages of guinea pigs, treated with a therapeutically active compound, exhibited increased phagocytic activity and sera of the same animals stimulated the macrophages from the normal animal to an increased phagocytosis. This stimulation is quantitatively reflected in the date, showing that the percentage of non-phagocytizing macrophages is decreased from 30% to 10% and the percentage of macrophages ingesting more than 10 BCG is increased from 10% to 30%. It seems that this method permits a more accurate recognition of stimulating agents than does the skin reaction method.

Correlation between the Chemical Structure and RES-stimulation Activity of Tested Compounds

All compounds tested are soluble only in lipoid or organic solvents. It seems that the lipophylic characteristic of the compounds is important for their activity.

In earlier experiments (5), striking differences have been found regarding the relation of chemical structure of bicyclodecapentane derivatives to the

India ink storage. Plattner (11) grouped these derivatives into three groups according to their absorption capacities in the visible spectrum. Only the members of the first group (chamazulene, guajazulene) induced local India ink storage. A characteristic of these compounds is that the carbon atoms in the second and sixth positions are not substituted.

In further experiments (8, 9), it was interesting to note that certain volatile oil components had no antituberculous activity (citronellol, linalool, vetiverol, terpineol, nerolidol, geraniol) but their acetates were therapeutically active (citronellyl acetate, linalyl acetate, vetivert acetate, terpenyl acetate, neryl acetate, geranyl acetate). It is evident that the RES-stimulating activity is related to the acetate radical. For instance, in the case of rhodinol and citronellol, the butyrate is less active than the acetate and the formate and phenyl acetate are completely inactive. The same relation can be seen in structurally unrelated compounds, such as terpineol and borneol; where the acetates are therapeutically active, the formate is inactive. On the other hand, however, cinnamyl acetate is less active than cinnamic alcohol.

TABLE III

ANTITUBERCULOUS ACTIVITY OF COMPOUNDS COMPARED WITH INDIA INK AND TRYPAN BLUE STORAGE IN THE SKIN OF RATS

Tested compounds	TB index	India ink	Trypan blue
Methyl heptenone	50	+++	+++
Methyl heptyl ketone	22	+++	++
Ethyl amyl ketone	34	++	±
2,6-Dimethyl-2-octen-8-al			
Citronellal	20.12	+++	+++
2,6-Dimethyl-2-octen-8-ol			
Citronellol (rhodinol)	75	±	±
Citronellyl acetate	19.13	+++	+++
Citronellyl butyrate	43	+	±
2,6-Dimethyl-2-octen-ol			
Rhodinol (citronellol)	96	−	−
Rhodinyl acetate	22	+++	+++
Rhodinyl phenyl acetate	80	±	±
Rhodinyl formiate	88	+	+
2,6-Dimethyl-2,6-octadien-8-ol			
Geraniol	77	+	+
Geranyl acetate	35.58	+++	+++
Trans-2,6-dimethyl-1,6-octadien-8-ol			
Nerol	63	+	+
Neryl acetate	24	++	++
2,6-Dimethyl octan-8-ol			
Pelargol	−	+	+
3,7-Dimethyl-1,6-octadien-3-ol			
Linalool (linalyl alcohol)	20	+	++
Linalyl acetate	25.3	++++	++++
Geraniol (citral)	53	++	++

NOTE: Legend as for Table I. Macrophage-stimulating effect of these groups of compounds was not tested.

It was of further interest to compare the therapeutic activity of some acyclic monoterpenoids with their chemical structure. The skeleton of these compounds is the methyl heptenone. Table III shows the index of tuberculosis in the guinea pig and India ink and trypan blue storage in rats after treatment with methyl heptenone, methyl heptyl ketone, ethyl amyl ketone, and related compounds. In the group of acyclic monoterpenoids, one of the secondary alcohols (citronellal) is active, the other one (geraniol) is inactive. Primary alcohols are in general inactive (citronellol, nerol, geraniol, pelargol) but the acetates are biologically active (the acetate of pelargol could not be obtained). Again linalyl acetate is as active as linalyl alcohol.

Among the monocyclic hydrocarbons, limonen showed the greatest therapeutic action. Phellandren, *p*-cimen, terpinolen, and terpineol were inactive, but limonen induced an intense India ink storage and showed good therapeutic activity on the experimental tuberculosis. A high index of tuberculosis was obtained after treatment with terpinolen and terpineol but terpenyl (1-methyl-4-isopropyl-1-cyclohexen-8-ol) acetate was therapeutically active.

Discussion

Alterations in the capillary system are generally considered to be the main features of the inflammatory process. An increased capillary permeability is induced at the beginning and maintained during the inflammation. The principal defence apparatus, is, however, an efficient RES which can eliminate *de facto* the noxious injury. This problem has been recently reviewed by the present authors (8).

The India ink and trypan blue reactions are indicators of increased capillary permeability. Neither of these two reactions is positive without an increase of capillary permeability. Both reactions, however, reflect the phagocytic activity of the endothelial cells, which becomes active as soon as particulate matter reaches the tissues (8).

Among the volatile oils and their components, a series of compounds were recognized which has therapeutic activity against experimental tuberculosis of the guinea pigs, when used alone or combined with subeffective doses of DSM. The therapeutically active compounds had no antituberculous effect *in vitro*. It has been postulated that the compounds act on the host. With the help of the India ink and trypan blue test, experimental evidence has been presented which indicates that the therapeutic activity of the compounds increases directly or indirectly with the capillary permeability at the site of application. Phagocytic activity of macrophages was stimulated *in vitro* by the active compounds. Macrophages from guinea pigs treated with the active compounds exhibited a stimulated ingestion of tubercle bacilli (BCG). Macrophages of normal guinea pigs were similarly stimulated by sera from the treated animals.

The volatile oils showing antituberculous activity also stimulate the two components of the inflammatory process which are the most important for the natural defence, for the functioning of the RES, and for the capillary

system. At the start of an inflammation, these important functions are maintained by histamine and 5-hydroxytryptamine (5-HT), which are endogenous substances, causing increased capillary permeability, built into the cells. During the inflammatory process, polypeptides are continually produced to maintain further the increased capillary permeability for influx of the defence elements into the damaged area. It is known from the experiments of Spector (12) that similarly acting peptides are continually formed during inflammation.

The compounds described in this study can maintain an increased capillary permeability as long as they remain in the organism, and can replace the action of substances which in the tissues are produced by complicated pathways to maintain the increased capillary permeability. The compounds probably do not act by the liberation of histamine or 5-HT since the India ink and trypan blue reactions occur also in the histamine depleted animals (13) after percutaneous or intramuscular administration of the compounds. It can be supposed, therefore, that the described compounds induce the reactions necessary for the defence processes in inflammation by acting directly on the capillaries.

Another characteristic feature of the compounds is the stimulation of the macrophages. This action would appear important in influencing beneficially the outcome of chronic inflammation. Since the experiments of Jancsó (14) it is known that endothelial cells of the loose connective tissue acquire a phagocytic capacity as soon as particulate matter is present in the blood or intercellular space. It can be postulated that the compounds which stimulate the macrophages stimulate similarly all mobile and fixed endothelial cells to an increased phagocytic activity, since there is no genetic difference among endothelial cells. However, further experimental proof is required to ascertain whether percutaneous administration of the compounds simply increases the capillary permeability, or stimulates also the histiocytes, fibroblasts, and mobile macrophages.

The activity of the compounds is associated with their lipophylic character and their chemical structure. Active compounds were found among components of volatile oils and experiments with other groups (phenyl methyl ethers) were negative. Most of the active compounds were found among the acyclic and monocyclic carbohydrates. The differences in the stimulating activity among structurally related compounds are instructive. The primary alcohols show activity, the secondary none. Among isomeric compounds the one is active, the other is not. In several chemically unrelated groups an inactive compound becomes active if acetylated.

Recently Heller *et al.* (15) studied the effect of natural and synthetic estrogens on the function of the RES and obtained similar results. They found among the estrogens compounds which had a powerful stimulatory effect on the phagocytic velocity of the RES. If structural changes were made in the molecules of effective compounds this change led to a loss of activity. On the other hand inactive compounds increased the RES activity if specific groups were introduced into the chemical structure.

The fact that small chemical alterations may increase the activity of a compound is encouraging and suggests the necessity of further systematic studies on this field.

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PURIFICATION AND PROPERTIES OF 2,5-DIKETOGLUCONIC ACID DECARBOXYLASE[1]

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Abstract

2,5-Diketogluconic acid decarboxylase has been purified from extracts of *Acetobacter melanogenum*. This paper reports the method of purification, the properties of the purified enzyme, and some properties of the reaction product. A crystalline *o*-nitrophenylhydrazone derivative of the decarboxylation product has been obtained.

Introduction

It has been shown by Katznelson *et al.* (3) that aged intact cells of *Acetobacter melanogenum* oxidize glucose to gluconic, 2-ketogluconic, and finally to 2,5-diketogluconic acids with a total oxygen consumption of 1.5 μM. per μM. of glucose. With fresh cells they obtained additional oxygen consumption. It has been observed recently (1) that 2,5-diketogluconate may be further oxidized in the presence of phenazine methosulphate by cell-free extracts of *A. melanogenum* with the accumulation of α-ketoglutarate. A "pentose-like" compound was found to be an intermediate in the conversion of 2,5-diketogluconate to α-ketoglutarate. It was suggested in the above paper that 2,5-diketogluconate is first decarboxylated to a five-carbon compound, which on further oxidation gives rise to α-ketoglutarate. The present communication deals with the purification and study of the properties of the enzyme responsible for the decarboxylation of 2,5-diketogluconate. Some attempts to identify the reaction product are also described.

Methods

Preparation of 2,5-Diketogluconate

2,5-Diketogluconate was prepared by a modified method of Katznelson *et al.* (3). Calcium 2-ketogluconate (Nutritional Biochemicals, 3200 μM.) was dissolved in 20 ml. distilled water and dispensed into the side arms of four Warburg vessels (125 ml. capacity). The center wells of the vessels contained 1 ml. 20% KOH. A cell suspension of aged *Acetobacter melanogenum* (1 g. wet weight/3 ml. water) adjusted to pH 3.5 was placed into the main compartment. After temperature equilibration, 2-ketogluconate was added from the side arm and oxygen consumption measured. As soon as theoretical oxygen consumption was reached (0.5 μM. per μM. of 2-ketogluconate) the reaction mixture was removed from the vessels and centrifuged at 4° C. at 10,000 $\times$ *g* for 10 minutes. The supernatant was immediately dispensed into several ampoules and stored at $-30°$ C.

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[2]National Research Council of Canada Fellow in collaboration with the Canada Department of Agriculture, 1955–1957.

Enzyme Assay

The 2,5-diketogluconic acid decarboxylase preparation was assayed in the Warburg apparatus at 30° C. by measuring carbon dioxide production in an atmosphere of 95% nitrogen and 5% carbon dioxide. Enzyme activity is defined as the amount of carbon dioxide liberated per mg. of protein in the first 20 minutes following addition of the substrate. Protein was estimated by the method of Lowry *et al.* (5).

The vessels contained 0.5 ml. 2,5-diketogluconate (20 μM.) in the side arm and 1.0 ml. enzyme, 0.3 ml. manganese chloride, $MnCl_2$ (0.01 *M*), and 0.5 ml. of 0.03 *M* citrate–phosphate buffer (6) at pH 3.5 in the main compartment. Distilled water was added to bring the final volume to 3.0 ml. The vessels were gassed with 95% nitrogen and 5% carbon dioxide mixture for 10 minutes and the substrate was added after a further 10 minute equilibration period with the stopcocks closed.

Results

Enzyme Preparation

Cells were grown and harvested as described previously (1). The packed, washed cells of *A. melanogenum* were suspended in water (1 g. of wet weight of cells per 3 ml.) and treated with sonic vibration in a Raytheon 10 kc. magnetostrictive oscillator for 5 minutes. The mixture was then centrifuged for 15 minutes at 10,000 × *g* at 4° C. The residue was discarded.

The supernatant (30 ml., pH 5.6) was chilled and treated with 5.3 g. of solid ammonium sulphate (30% saturation) for 10 minutes. The temperature was kept at 0–2° C. with the aid of an ice bath and all subsequent steps were carried out at that temperature. Following centrifugation at 10,000 × *g* for 15 minutes, the residue was discarded; the supernatant (25 ml.) was then treated with 6.8 g. of solid ammonium sulphate (70% saturation). After 10 minutes the mixture was centrifuged at 10,000 × *g* for 15 minutes. The supernatant was poured off and the residue dissolved in 15 ml. distilled water.

The pH of the dissolved residue was usually 5.0. The latter was treated with cold 0.1 *M* citric acid to bring the pH to 3.0 and the resulting precipitate centrifuged down at 10,000 × *g* for 15 minutes. The residue was discarded and the supernatant treated with an approximately 1/3 volume of calcium phosphate gel (Sigma Chemical Co., containing 12.4 mg. calcium phosphate per ml.) with gentle stirring for *exactly* 2 minutes. Centrifugation was then carried out at 13,000 × *g* for 15 minutes. The residue was again discarded and the supernatant used as the enzyme preparation. Longer periods of contact of the supernatant with calcium phosphate gel resulted in preparations with lower enzyme activity. The resultant enzyme solution was found to be 28-fold purified as compared with the original sonic extract (Table I) on the basis of protein content.

Freezing the cells or the extract in a commercial freezer at any step in the purification procedure caused inactivation of the enzyme. Furthermore, treatment with acetone at 0° C., heat at various temperatures, protamine

sulphate or manganese chloride always resulted in considerable loss in enzyme activity owing to adsorption by the precipitates so formed. These techniques were, therefore, unsuitable for purposes of concentrating the enzyme described here.

TABLE I

PURIFICATION OF 2,5-DIKETOGLUCONIC ACID DECARBOXYLASE

Stage of purification	Protein/ml., mg.	Activity/ml., μl.	Activity/mg. protein
Supernatant from centrifuged sonic extract	50.0	65.0	1.3
30–70% ammonium sulphate fraction (ppt. dissolved in water)	28.0	61.0	2.2
Supernatant after precipitation at pH 3.0	4.1	32.0	7.8
Supernatant after 2 minute calcium phosphate gel treatment	1.4	51.0	36.4

Properties of the Enzyme

The purified enzyme decarboxylates 2,5-diketogluconate with the production of 1 μM. carbon dioxide per μM. of 2,5-diketogluconate. Freezing of the enzyme solution in a freezer causes inactivation. The optimum pH was found to be between 3.0 and 3.5 (Fig. 1), although this pH is not favorable for storage of the enzyme. The enzyme may be stored in the refrigerator for about one week without appreciable loss in activity provided the pH is kept near 6.0. Dialysis for 24 hours or more causes considerable loss of activity which is not restored by the addition of thiamine pyrophosphate (ThPP), magnesium ions (Mg^{++}), or manganese ions (Mn^{++}) alone or in

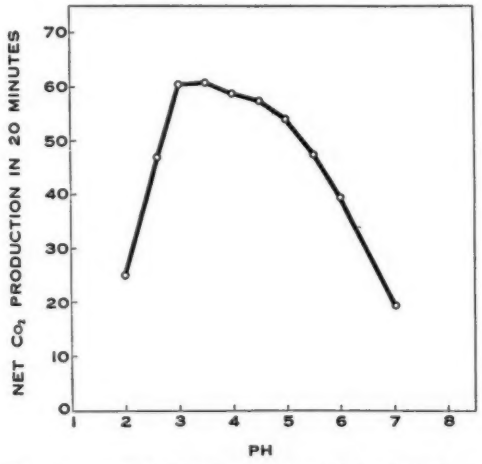

FIG. 1. The pH optimum of 2,5-diketogluconic acid decarboxylase.

combination. The addition of a boiled, crude extract to the dialyzed preparation has no stimulatory effect. The undialyzed enzyme is not stimulated appreciably by ThPP but is stimulated considerably by Mn^{++} (Table II). When both ThPP and Mn^{++} are added together the activity is less than when Mn^{++} is added alone.

Diphosphopyridine nucleotide, triphosphopyridine nucleotide, adenosine triphosphate, adenosine diphosphate, adenosine monophosphate, pyridoxal phosphate, glutathione, and coenzyme A did not stimulate.

TABLE II

EFFECT OF THIAMINE PYROPHOSPHATE AND OF MANGANESE IONS ON THE DECARBOXYLATION OF 2,5-DIKETOGLUCONATE

System	CO_2 production in 60 min., μl.
Enzyme	72
Enzyme + ThPP	86
Enzyme + Mn^{++}	154
Enzyme + Mn^{++} + ThPP	105

NOTE: Components of reaction mixture: see under "enzyme assay"; Mn^{++} 0.001 *M*, ThPP 1 mg. per vessel.

The enzyme preparation decarboxylated α-ketoglutarate very slightly. Gluconate, 2-ketogluconate, acetate, malate, oxalacetate, succinate, citrate, and phenylalanine were not decarboxylated. Considerable decarboxylation of pyruvate took place with the purified enzyme preparation in the presence of added Mn^{++} but this was found to be dependent upon, and the rate proportional to, ThPP addition. This is in contrast to the 2,5-diketogluconate decarboxylation in which ThPP actually caused an inhibition when the system was operating under optimum conditions in presence of Mn^{++}.

After the anaerobic decarboxylation, the reaction mixture was treated with trichloroacetic acid. When aliquots of this trichloroacetic acid extract were applied to filter paper, developed in butanol – acetic acid – water (4:1:2.5), and sprayed with aniline oxalate or phthalate, a new pink spot (R_f, 0.25) appeared. No such spot was detectable when the reaction was carried out with pyruvate or α-ketoglutarate as substrates.

Properties of the Product

The product had an R_f value not unlike that of arabinose (0.24) in the above solvent system. Its R_f in *n*-propanol – methyl benzoate – formic acid – water (100:75:35:15) was 0.24 as compared to 0.23 for arabinose. In phenol–water (4:1) the product and arabinose were well separated, the former having an R_f value of 0.71 and the latter of 0.59. Specific sprays for ribulose (2), xylulose (2), and sedoheptulose (4) when applied to the chromatograms yielded negative results, suggesting that the product did not correspond to any of these substances. Dihydroxyacetone was also ruled out chromatographically.

The product of reaction gave a positive orcinol reaction (7) but the spectrum of the product of the orcinol reaction was different from those given by xylulose and arabinose (Fig. 2). It had a typical pentose peak at 670 mμ, a hump at 540–550 mμ similar to that of the ketopentoses (see curve 3), and another strong absorption band with an apparently characteristic peak at 452 mμ. When eluted from paper chromatograms, the chromatographically purified product gave the same spectral characteristics after reaction with orcinol as did the crude preparation.

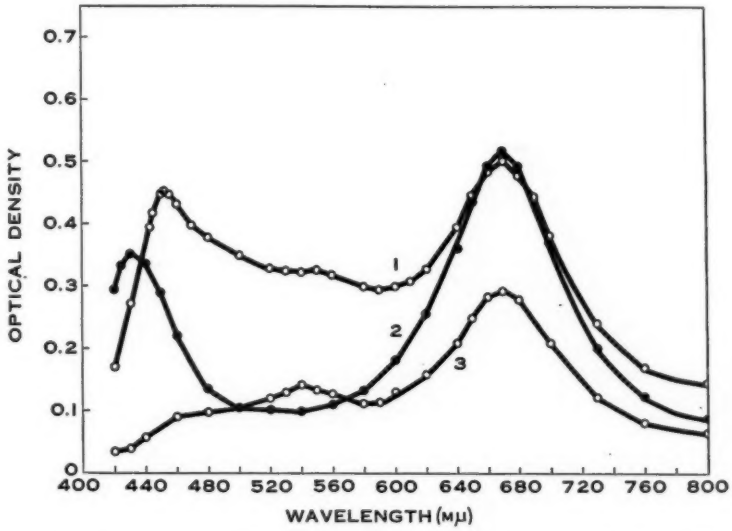

FIG. 2. Spectra of the decarboxylation product (Curve 1, estimated concentration: 30 μg.), arabinose (curve 2, 30 μg.) and xylulose (curve 3, 20 μg.) following their reaction with orcinol and ferric chloride.

Preparation of a Derivative

The reaction mixture obtained from Warburg vessels containing an estimated 30 mg. of reaction product was lyophilized in a series of ampoules as quickly as possible. The contents of each vial were suspended in 2 ml. ethanol and 0.2 ml. water and centrifuged in a clinical centrifuge for 5 minutes. Thirty milligrams of freshly sublimed *o*-nitrophenylhydrazine, dissolved in 1 ml. ethanol and containing two or three drops of glacial acetic acid, was added to the supernatant at room temperature. Hydrazone formation began at once and the mixture was allowed to stand at room temperature for 2 hours. The volume was reduced to 1/3 of the original by evaporation with an electric fan. The product was then filtered on a sintered glass funnel, washed three times with cold ethyl acetate, and dried in a vacuum desiccator over calcium chloride. It was recrystallized once from ethanol, the recrystallized hydrazone giving a sharp melting point at 181° C. The crystals

appeared as long, yellow needles under the microscope. The melting point of pure *o*-nitrophenylhydrazine was 93° C. Further microanalytical studies are now under way in an effort to identify the product more precisely.

Discussion

Purification of the decarboxylation enzyme has now made possible a more detailed study of the factors involved in the formation of α-ketoglutarate from 2,5-diketogluconate by extracts of *A. melanogenum*. However, several serious difficulties still exist which must be overcome before this problem may be advanced materially. The decarboxylation product is extremely sensitive to heat and thus both derivative formation and attempts at removal of the hydrazine moiety from the derivative must be carried out at room temperature. This has made the chemical aspects of the study on the derivative a difficult task. So far, not enough material has been available to permit a detailed structural study to be carried out. It is hoped, however, that work now in progress will soon permit the final identification of the product.

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THE EFFECT OF MUSCULAR EXERCISE ON PLASMA SODIUM AND POTASSIUM IN THE RAT[1]

F. A. Sréter and Sydney M. Friedman

Abstract

After running a distance of 100 meters in 7 minutes, untrained rats showed a rise in plasma potassium and a fall in plasma sodium as measured in tail vein samples. These changes are in accord with *in vitro* observations of the effects of exercise on isolated muscle preparations and similarly are taken to indicate a gain of sodium and a loss of potassium by the exercised muscles in the whole animal. Within 10 minutes of completion of the exercise, plasma sodium concentration was restored to normal while potassium was restored within 20 minutes. Exercise was accompanied by a fall in haematocrit, which remained low for up to 40 minutes. A period of 2 months of preliminary training modified the response to exercise. In these trained animals, a fall in sodium concentration occurred as before but the rise in potassium concentration was less in degree and the haematocrit did not change. It is suggested that the *rate* of increase of plasma potassium is an index of muscle efficiency while the *height* of plasma potassium is correlated with the fatigue limit of exercise.

Introduction

Stimulation of the motor nerve to an isolated skeletal muscle fiber releases acetylcholine which depolarizes the fiber in the region of the motor end plate (2, 3). During the depolarization process the permeability of the muscle membrane to sodium is increased and this is followed by a movement of potassium ions outward. In experiments on isolated whole muscle preparations these changes are reflected in a diminution of sodium and an increase in potassium in the medium or perfusion fluid (6, 8). Fleckenstein (4) has suggested that the ion changes are primary initiators of the ensuing chemical changes of contraction.

So far, changes in the perfusate or medium during muscular activity appear to have been studied only in isolated preparations. The present studies were designed to determine whether these same shifts were discernible in the whole animal during gross muscular exercise. These experiments form the first part of an investigation into the relation of steroid hormones to muscular efficiency.

Methods

Adult male albino rats of an inbred Wistar strain, 300–350 g. in weight, were used throughout. Blood samples, 0.7–0.8 ml., were drawn without anaesthesia from the tail vein using a 22 gauge needle and heparin-moistened syringe. Samples were obtained without exciting the animal within 1–2 minutes. The animals were exercised on a 70 cm. treadmill with adjustable speed and a counter was used to determine the amount of performed work as distance in meters. Training, as required, was carried out by exercising animals for 6 to 8 minutes daily for 2 months.

Plasma was analyzed for sodium and potassium by flame photometry.

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Contribution from the Department of Anatomy, The University of British Columbia, Vancouver, B.C., Canada. This work was carried out with the aid of a grant from the Muscular Dystrophy Association of Canada.

Experimental

Experiment 1. Plasma Na and K Immediately after Exercise in Untrained Rats

Three days before the experiment a series of rats was tried out on the treadmill in order to select a group of runners. (In our experience some rats are unwilling to perform on the treadmill.) From these, eight rats were selected to serve as test group (exercised) and eight as controls. On the experimental day, the test group was made to run 100 meters in 7 minutes. A blood sample was taken immediately prior to and immediately after the exercise period. Similar blood sampling was carried out in the control group. Table I presents the findings.

TABLE I

THE EFFECT OF 7 MINUTES OF CONTINUOUS RUNNING ON PLASMA Na AND K AND ON HAEMATOCRIT IN UNTRAINED RATS

Group	No. of rats	Na, meq./liter		K, meq./liter		Haematocrit, %	
		Before	After	Before	After	Before	After
Control, non-exercised	8	150.2±1.7	148.6±1.3	4.82±0.29	4.87±0.22	48.9±0.7	48.0±0.8
Test, exercised	8	149.4±0.9 *	146.3±0.8	4.60±0.15 **	5.33±0.05	51.2±0.8 **	48.9±0.7

NOTE: Tail blood samples were taken immediately before and after the exercise. Samples were taken at the same intervals in the controls. ± =standard error.
* = $p < 0.05$.
** = $p < 0.02$.

The exercised group showed a significant fall in haematocrit, a change which actually was evident in seven of the eight animals in the group. The small fall which occurred in the control group was presumably due to the drawing of the first blood sample.

Sodium concentration similarly showed a significantly greater fall in the exercised group than could be referred to the blood sampling procedure.

The clearest change was in the potassium concentration which rose in every exercised rat so that the average potassium value for the group rose from 4.60 to 5.33 meq./liter while no effect was observed in the control group.

Experiment 2. Recovery of Plasma Na and K Levels after Exercise in Untrained Rats

The experimental plan was basically similar to that of the previous experiment in that test groups of exercised rats were compared with control groups of unexercised animals. The essential difference was that the two blood samples were both taken from the test group *after* completion of the exercise, the first immediately after and the second a chosen interval later. The experiment was carried out in five sections, all beginning at the time of completion of the exercise but ending either 10, 20, 30, 40, or 50 minutes later. The findings are presented in Table II.

The fall in plasma Na concentration which followed exercise in the first experiment occurred again here. The first or basal Na measurement in all

TABLE II

THE RECOVERY RATE FOR PLASMA Na AND K AND HAEMATOCRIT IN UNTRAINED RATS AS MEASURED FOR VARIOUS INTERVALS BEGINNING IMMEDIATELY AFTER EXERCISE (0 MINUTES). SYMBOLS AS IN TABLE I

Interval	Group	No. of rats	Na, meq./liter		K, meq./liter		Haematocrit, %	
			Sample 1	Sample 2	Sample 1	Sample 2	Sample 1	Sample 2
0 – +10 min.	Control	8	149.2±1.2	148.9±1.0	4.81±0.05	4.79±0.06	47.8±0.7	48.1±0.8
	Test	8	147.9±0.8	149.3±1.0	5.36±0.09 **	5.06±0.07	48.8±0.7 **	46.3±0.4
0 – +20 min.	Control	8	150.3±1.5	149.8±1.4	4.85±0.11	4.78±0.08	48.9±1.0	48.5±1.0
	Test	8	147.2±1.7	148.8±2.3	5.30±0.16 **	4.81±0.09	50.3±1.0 **	46.7±1.3
0 – +30 min.	Control	6	151.2±1.2	149.6±0.7	4.80±0.10	4.77±0.09	48.6±1.3	49.3±1.3
	Test	8	148.9±1.5	151.0±1.2	5.52±0.15 **	4.79±0.06	49.2±0.8 **	47.2±1.2
0 – +40 min.	Control	6	150.7±0.4	150.2±2.1	4.74±0.06	4.79±0.06	49.0±0.9	49.6±1.2
	Test	6	148.6±1.1	149.8±1.1	5.35±0.16 **	4.62±0.07	51.6±1.4 *	48.8±1.6
0 – +50 min.	Control	8	151.6±0.9	149.6±0.5	4.79±0.08	4.88±0.11	48.1±0.7	49.2±0.6
	Test	8	150.0±0.7 †	151.0±1.6	5.37±0.11 ** †	4.71±0.09	50.4±0.9 ** †	48.6±0.4

†Note consistent trend in this column between control and test group.

five sections of this experiment was in every case lower in the test group than in the corresponding control, a significant finding. Thus, the fall in Na caused by exercise which was observed in the internally controlled arrangement of the previous experiment was here reproduced consistently (by differences of 1.3 to 3.1 meq./liter) in an externally controlled situation. The Na concentration was, however, completely restored to normal within 10 minutes of the exercise.

The significant rise in plasma K concentration observed in the previous experiment was also consistently reproduced in the comparison of the first or basal K measurement of test and control groups here. The elevated plasma K had fallen 10 minutes after exercise but was still significantly elevated in comparison with the control group. The second K value, measured at later intervals, was completely normal.

The haematocrit was lower at all intervals after exercise in the test group. The basic findings of Experiments 1 and 2 are presented graphically in Fig. 1.

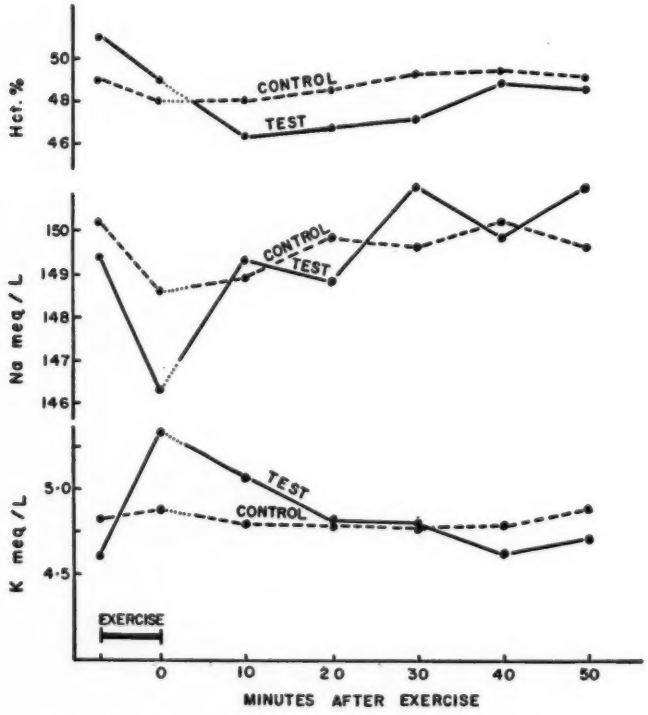

FIG. 1. Composite graph showing the effect of exercise on plasma Na and K concentrations and haematocrit and subsequent recovery. The absolute values are drawn from Tables I and II.

Experiment 3. Plasma Na and K Immediately after Exercise in Trained Rats

Nineteen rats, trained as described above for 2 months, were divided into two groups, according to their running abilities. The best 10 served as test group, the remaining nine as controls. The test group was subjected to a period of exercise as in Experiment 1, a blood sample being taken immediately before and immediately after the period of exercise. Comparable blood samples were taken from the controls without any exercise. The results are presented in Table III.

TABLE III

THE EFFECT OF 7 MINUTES OF CONTINUOUS RUNNING ON PLASMA Na AND K AND ON HAEMATOCRIT IN TRAINED RATS. SYMBOLS AS IN TABLE I

Group	No. of rats	Na, meq./liter		K, meq./liter		Haematocrit, %	
		Before	After	Before	After	Before	After
Control, non-exercised	9	146.9±1.3	146.3±1.8	4.77±0.11	4.84±0.09	48.6±1.4	48.1±1.0
Test, exercised	10	148.4±1.1 **	145.1±0.8	4.81±0.11 *	5.10±0.10	50.1±0.8	50.4±0.5

In contrast to the preceding experiments, no change in haematocrit was observed following exercise in the trained test group. As before, plasma Na fell and K rose after exercise but here, while both the Na and K shifts were well defined, the rise in K was less in degree than in previous experiments with untrained animals.

Discussion

Although *in vitro* studies have established the importance of sodium and potassium in muscle contraction (1, 7) few direct *in vivo* observations have been carried out to determine whether these ion changes are reflected in changes in blood concentrations. Grob *et al.* (5) have recently attempted this for potassium and have shown that muscular activity in the forearm in man is in fact followed by a movement of potassium out of the muscles as demonstrated by an increase in the arteriovenous difference.

The present experiments show that during muscular effort in the rat there is a net loss of sodium and a gain of potassium in the medium, here represented by the plasma. It is reasonable to assume that the converse changes occur in the muscles. This would not be expected to occur if the restoration of ionic gradients were completed between contractions. Accordingly then, what might be termed sodium and potassium "debts" are incurred during the period of exercise.

The accumulation of sodium within the muscle cells, or the increase in potassium outside (or both) mean, in themselves, progressively altered ionic gradients as exercise continues and these ultimately must limit the ability to perform work despite the continued stimulus. In short, the ion debt must be an important factor in fatigue. This view is supported by the fact that the trained rats did not show as great an increase in plasma potassium as did the untrained animals.

If these views are correct the *rate* of potassium rise during exercise would represent an index of muscle efficiency while the *height* of potassium rise would be correlated with fatigue. It is well known, of course, that conditions associated with a high plasma potassium concentration are often accompanied by a ready fatigability.

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FURTHER TOXICOLOGICAL AND BIOCHEMICAL STUDIES ON ω-FLUORO COMPOUNDS[1]

I. G. Walker and J. M. Parker[2]

Abstract

The toxicity to mice of the 5-fluoroamyl halides and six 1-fluoroalkanes (C_6–C_{11}) is reported. Unlike the other odd-numbered ω-fluoroalkyl halides the 5-membered compounds are quite toxic. In the 1-fluoroalkane series as in other ω-fluoro series the even-numbered compounds are the more toxic. The 5-fluoroamyl halides and 9-fluorononyl chloride, injected into mice in doses of three to four times the LD_{50}, induce an increase in the citrate content of the kidneys between 20 and 90 minutes after injection. For the even-numbered ω-fluoro compounds, a pronounced accumulation has already developed by 20 minutes. Both the even and odd-numbered 1-fluoroalkanes cause an increase in the citrate content of the kidneys of mice injected with them. However, the former compounds promote a more rapid elevation of the citrate content. Other representative odd-numbered ω-fluoro compounds were tested for their ability to cause citrate accumulation at a time greater than 20 minutes. The lack of effect of these compounds on kidney citrate at this later time suggested that among the odd-numbered ω-fluoro compounds the ability to promote such a citrate accumulation is peculiar to the ω-fluoroalkyl halides and the 1-fluoroalkanes.

Introduction

Pattison and co-workers (see (3) for references to their publications) have synthesized many homologous series of ω-fluoro compounds in which the members exhibited an alternation in toxicity which depended on the number of carbon atoms in the chain. The even-numbered compounds were always considerably more toxic than the odd-numbered compounds. It was demonstrated also by Parker and Walker (3) that a representative even-numbered member from each of several series when administered to mice caused an increase in the citric acid content of the kidneys. A representative odd-numbered member of the corresponding series did not cause this accumulation. 5-Fluoroamyl fluoride, bromide, and iodide, for which only approximate toxicity estimations were available, appeared not to fit the general toxicity pattern demonstrated by Pattison (6). They appeared to be more toxic than other odd-numbered ω-fluoroalkyl halides. We therefore have made more accurate toxicity estimations for these compounds and have investigated the effect of these compounds on the kidney citrate content of mice injected with them. The 1-fluoroalkanes, a new class of ω-fluoro compounds, were studied in the same manner.

Experimental

The experimental procedures have been described previously (3). Briefly, they were as follows:

Toxicity estimations were made on Connaught strain white mice. The compounds were dissolved in propylene glycol and administered by intra-

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peritoneal injection, in volumes not exceeding 0.1 ml. The LD_{50} values for which 95% confidence limits are given were calculated by the method of probit analysis. The other approximate LD_{50} values were obtained graphically. To ascertain if a given compound caused citric acid accumulation, mice were given an intraperitoneal injection of the compound dissolved in propylene glycol. Because of the insolubility of 9-fluorononyl chloride in non-toxic solvents, this compound was injected directly without diluting. The dose for all compounds was three to four times the LD_{50} unless otherwise stated. The animals were sacrificed at the times indicated and the citric acid content of their kidneys was estimated as previously described (3), with the modifications noted below.

In previous experiments with fluoroacetate and 6-fluorohexylamine (4), and in some unpublished studies with 5-fluoroamyl iodide, it had been found consistently that citrate accumulation was greater in the kidneys than in the other organs studied (liver, spleen, and brain). Kidney was therefore chosen for citrate analysis in the present work, in order to facilitate the demonstration of any citrate accumulation which might occur. A large dose, three to four times the LD_{50}, was used for the same reason.

Modifications in Method for Estimating Citric Acid

Centrifugation after shaking of the pentabromacetone solution with petroleum ether was found not to be necessary. An aqueous 4% sodium sulphide solution was used in place of the aqueous pyridine – sodium sulphide solution. Photometry was performed with a Beckman DU spectrophotometer instead of a Klett photometer, and therefore, ungraduated centrifuge tubes were used instead of Klett tubes when the petroleum ether extract was shaken with the sodium sulphide solution. The resulting colored aqueous layer was transferred by pipette to 1 cm. pyrex cuvettes for reading in the Beckman at a wave length of 465 mμ. When the amount of citrate in the kidney extract was small, the standards were made to contain 0, 20, 40, and 60 μg. of citric acid. At first 10 animals were used for each determination, their kidneys being pooled. Later, five animals were used, in which case the volume of the kidney extract was reduced by one half.

Results and Discussion

In Table I are shown the toxicities of the 5-fluoroamyl halides. By way of comparison the available figures for the toxicities of other odd-numbered ω-fluoroalkyl halides are all greater than 0.40 mM./kg. while the available values for even-numbered ω-fluoroalkyl halides range from 0.014 to 0.16 mM./kg. These values have been calculated from the data reported by Pattison (6). It is evident from this comparison that the five-membered ω-fluoroalkyl halides are more toxic than other odd-numbered ω-fluoroalkyl halides. Indeed, apart from 5-fluoroamyl chloride they are as toxic as the even-numbered ω-fluoroalkyl halides. This finding does not fit the general toxicity pattern exhibited by ω-fluoro compounds as shown by Pattison (5, 6). That is, in an homologous series of ω-fluoro compounds, those members

which contained an even number of carbon atoms in their chain were more toxic than those which contained an odd number. It was also shown by Parker and Walker (3) that representative even-numbered ω-fluoro compounds, when administered to mice at a dose which was three to four times the LD_{50}, had promoted a marked increase in the citrate content of the kidney 20 minutes after dosage. Representative odd-numbered compounds when tested in the same manner had no effect on the kidney citric acid content. We were prompted to test the effect of the 5-fluoroamyl halides on the citrate content of the kidney to see if these compounds differed in this effect from other odd-numbered ω-fluoroalkyl halides, and the result is shown in Table II. The second column of Table II shows the citrate concentration in the kidneys of mice 20 minutes after injection with a dose of 5-fluoroamyl halide which was three to four times the LD_{50}. These citrate concentrations are just above the normal range, with one exception, which is within the normal range. Previously, with other ω-fluoro compounds, this type of test had yielded an unequivocal result. The kidney citrate was either well within the normal range or was greatly in excess of the normal (3). Thus, we were prompted to repeat the experiment but with the citrate analyses being performed at a greater time interval after administration of the fluoro compounds. The slight degree of citrate accumulation at 20 minutes suggested that the 5-fluoroamyl halides might bring about a relatively slow citrate accumulation which would show up at some time greater than 20 minutes. The

TABLE I

THE TOXICITIES OF THE 5-FLUOROAMYL HALIDES

The 5-fluoroamyl halides	LD_{50} (95% confidence limits), mM./kg.
5-Fluoroamyl fluoride	0.147 (0.075–0.285)
5-Fluoroamyl chloride*	0.244 (0.165–0.358)
5-Fluoroamyl bromide*	0.062 (0.048–0.080)
5-Fluoroamyl iodide	0.036 (0.031–0.042)

*Estimated at Suffield Experimental Station, Ralston, Alta.

TABLE II

CITRATE CONCENTRATION IN KIDNEY AFTER ADMINISTRATION OF THE 5-FLUOROAMYL HALIDES

Compound	Citrate concentration in kidney*, μg./g.	
	20 min. after injection	90 min. after injection
5-Fluoroamyl fluoride	135	730
5-Fluoroamyl chloride	114	232
5-Fluoroamyl bromide	62.8	862
5-Fluoroamyl iodide	149	888
Normal kidney citrate 74.9 μg./g.	S.D. = 25.9 μg./g. (seven estimations)	

*Each figure is a single estimation determined on the pooled kidneys from 10 mice.

TABLE III

CITRATE CONCENTRATION IN KIDNEY AFTER ADMINISTRATION OF ODD-NUMBERED ω-FLUORO COMPOUNDS

Compound	Approx. LD_{50}, mM./kg.	Time of death or sacrifice, min.	Citrate concn., μg./g.
3-Fluoropropionic acid	0.65	30–40	18, 23, 52
5-Fluoroamyl isothiocyanate	0.46	60–75	0, 0, 8
11-Fluoroundecanoic acid	0.28	90	10, 28
5-Fluoroamyl tosylate	>0.64	90	26, 130, 26
7-Fluoroheptylamine*	0.38	90	100
9-Fluorononyl chloride	1.09	90	980, 630
Normal kidney citrate 74.9 μg./g. S.D. = 25.9 μg./g. (seven estimations)			

*Dose was two times LD_{50}.

third column of Table II shows that when the animals were sacrificed at 90 minutes the citrate content of the kidneys was markedly elevated. The time, 90 minutes, was chosen quite arbitrarily. It was considered long enough to allow for any metabolic process which the fluoro compound might undergo and it usually assured that the mice were still alive. In our previous paper (3) kidney citrate analyses were performed only at 20 minutes after the administration of the ω-fluoro compounds. Citrate analyses at other times did not seem necessary. In view of the finding that the 5-fluoroamyl halides caused a marked citrate accumulation 90 minutes after administration, it became necessary to test other odd-numbered ω-fluoro compounds for their ability to induce citrate accumulation at 90 minutes. The compounds available for testing were 9-fluorononyl chloride, 3-fluoropropionic acid, 11-fluoroundecanoic acid, 7-fluoroheptylamine, 5-fluoroamyl tosylate, and 5-fluoroamyl isothiocyanate. The results of such a test with these compounds are shown in Table III.* It may be seen that with 3-fluoropropionic acid and 5-fluoroamyl isothiocyanate the animals died before 90 minutes and showed no citrate accumulation. Therefore, it is unlikely that these compounds are capable of causing such an accumulation. With 11-fluoroundecanoic acid, 5-fluoroamyl tosylate, and 7-fluoroheptylamine the animals were still alive at 90 minutes and did not show a citrate accumulation. The possibility remains that such an accumulation could be found at a later time. The only compound in this group which has brought about an accumulation of citrate within 90 minutes is 9-fluorononyl chloride. This result, coupled with those obtained with 5-fluoroamyl halides, suggests that the odd-numbered ω-fluoroalkyl halides, unlike other odd-numbered ω-fluoro compounds,

*For several of these compounds it was not possible to give a dose three to four times the LD_{50} and sacrifice at 90 minutes because the animals died too quickly. As indicated in the table the dose of 7-fluoroheptylamine was reduced to twice the LD_{50} and for 3-fluoropropionic acid and for 5-fluoroamyl isothiocyanate the time of sacrifice after injection was decreased. A few of the animals died spontaneously from the lethal effects of the fluoro compounds and when this occurred the kidneys were removed within 15 minutes of death. From the work of Buffa and Peters (1) it was considered that the post-mortem decrease in citric acid in this time would not be significant.

are capable of causing a fairly rapid citrate accumulation in the kidneys of mice injected with these compounds. It is recognized that some of the citrate concentrations in Table III are much lower than the normal value we report but the significance of this is not known. For several organs of the rat including kidney, Buffa and Peters (1) found the normal citrate content to vary from 0 to about 60 μg. of citrate per g. of tissue and our findings may only be a reflection of a rather wide normal variation or of an inability to estimate accurately such low citrate concentrations.

The interpretation of the citrate accumulation which appears most logical to us is that the compounds which bring about an accumulation are metabolized to fluoroacetic acid with its well known sequelae (8). A proof of this interpretation will require the identification of fluoroacetate or fluorocitrate in the tissues of the poisoned animal. If this interpretation is correct, then its implications regarding the metabolism of ω-fluoroalkyl halides are interesting. The formation of fluoroacetate from an ω-fluoroalkyl halide containing an even number of carbon atoms can be explained by assuming, as Pattison did (6), that hydrolytic dehalogenation to the corresponding alcohol occurs. The alcohol is oxidized to the carboxylic acid which by β-oxidation yields fluoroacetate. The only way in which an odd-numbered ω-fluoroalkyl halide can yield fluoroacetate is through a chain cleavage reaction and there are few such reactions known in biochemistry. An example is provided in the metabolism of alkyl benzenes by the rabbit (2).

The 1-fluoroalkanes were investigated in much the same way as the 5-fluoroamyl halides, and the results are presented in Table IV. First, it may be seen that there is an alternation in toxicity amongst these compounds with the even-numbered members being significantly more toxic. Second, all the compounds cause a citrate accumulation. A first determination of 20-minute citrate values in which a single estimate was made for each compound (Trial 1) indicated that the odd-numbered compounds brought about a lesser citrate accumulation than did the even-numbered compounds. A later determination in triplicate with the only available compounds, 1-fluorooctane and 1-fluorononane (Trial 2), again showed this difference

TABLE IV

THE TOXICITIES OF THE 1-FLUOROALKANES AND THEIR EFFECT ON KIDNEY CITRATE CONCENTRATION

Compound	LD_{50} (95% confidence limits), μM./kg.	Kidney citrate, μg./g.		
		20 min. after injection		90 min. after injection
		Trial 1	Trial 2	
1-Fluorohexane	16.3 (13.8–19.0)	610		—
1-Fluoroheptane	297 (266–399)	173		813
1-Fluorooctane	20.5 (18.0–23.3)	465	580, 720, 600	—
1-Fluorononane	125 (106–147)	275	520, 490, 500	903
1-Fluorodecane	10.6 (8.44–13.8)	510		—
1-Fluoroundecane	89.1 (75.3–104)	59.0		712

although not as strikingly. Using "Student's" *t*-test the difference is significant at $p = .05$. The odd-numbered compounds were tested for their effect on citrate levels at 90 minutes to see if they would produce a sizeable accumulation at a later time, and it is apparent that they did.

Thus, the odd- and even-numbered 1-fluoroalkanes differ from one another in their degree of toxicity and in the rate at which they induce citrate accumulation in the kidneys of mice poisoned by them. The citrate accumulation in the kidney may be explained by the assumption that these compounds are metabolized to fluoroacetate which, through conversion to fluorocitrate, inhibits the enzyme aconitase. If this assumption is correct then the possible metabolic origin of the fluoroacetate deserves comment. From an odd-numbered 1-fluoroalkane, a necessary step would appear to be a chain cleavage. The same mechanism could also produce fluoroacetate or substances easily metabolized to fluoroacetate from 1-fluoroalkanes which contained an even number of carbon atoms. In addition, an even-numbered 1-fluoroalkane could be expected to yield fluoroacetate if its terminal methyl group were oxidized to a carboxylic acid. This type of reaction would be analogous to the ω-oxidation proposed by Verkade (9) for the metabolism of the C_8 to C_{12} fatty acids and which has received support from the work of Weitzel (10).

The results of the present investigation raise many interesting questions which are deserving of further study. It remains to be established that ω-fluoro compounds which cause a citric acid accumulation in the tissues of animals poisoned with them do so through metabolic conversion to fluoroacetate. The citrate accumulation is only suggestive of such a mechanism. A second problem which arises from this work is that of the mechanism of toxicity of the various ω-fluoro compounds. In our previous paper (3) the finding that the toxic, even-numbered ω-fluoro compounds caused a citrate accumulation, while the relatively non-toxic, odd-numbered ones did not, was taken as evidence for the proposal that even-numbered compounds were metabolized to fluoroacetate, and therein resided their toxic nature. On the other hand, it was considered that the odd-numbered compounds were not very toxic because they were metabolized to fluoropropionic acid (7). In the present work, differences in toxicity among the various compounds were compensated for by using equitoxic doses. Despite this, some compounds killed rapidly without producing a citrate accumulation, some caused rapid citrate accumulation, and still others caused a delayed or no citrate accumulation. These findings imply at least two and possibly more mechanisms of toxicity. There may well be no general relationship between the toxicity of an ω-fluoro compound and the fact that it causes citrate to accumulate. A further question is, what is the nature of the effect of the halogen atom on the toxicity of the 5-fluoroamyl halides? If these points become clarified, the study of ω-fluoro compounds should yield valuable information regarding metabolic pathways of various compounds, about the effect of molecular substitution on toxicity and enzyme specificity, and on the general problem of intoxication.

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EFFECTS OF CATIONS ON SUGAR ABSORPTION BY ISOLATED SURVIVING GUINEA PIG INTESTINE[1]

E. RIKLIS[2] AND J. H. QUASTEL

Abstract

The rate of absorption of glucose from isolated surviving guinea pig intestine increases with increase of the concentration of glucose in the lumen until a maximum rate is obtained. The relation between absorption rate of glucose and initial glucose concentration conforms to an equation of the Michaelis–Menten type. The *apparent* K_m (half saturation concentration) is 7×10^{-3} *M*. Increase of the concentration of potassium ions in the Ringer–bicarbonate solution bathing the intestine leads to an increase of the rate of glucose absorption, this being most marked with 15.6 meq./liter K^+ and 14 m*M* glucose. No such stimulating action of potassium ions is observed on glucose absorption under anaerobic conditions. The effect of increased potassium ion concentration is to accelerate the rate of transport found with low concentrations of glucose to the maximum value found with high concentrations of the sugar. Sodium ions must be present for glucose absorption to take place and omission of magnesium ions from a Ringer–bicarbonate solution, containing 15.6 meq./liter K^+, brings about a decreased rate of active glucose transport. Magnesium ions are necessary for the stimulated rate of glucose absorption obtained in the presence of potassium ions. The presence of ammonium ions decreases the rate of glucose absorption. Potassium ions may be effectively replaced by rubidium ions for stimulation of glucose transport. Cesium ions do not activate. The proportion of glucose to fructose appearing in the serosal solution, when fructose is absorbed from the mucosal solution, depends on the concentration of fructose present. The proportion may be as high as 9:1 with low (7 m*M*) fructose concentrations; it decreases with increasing fructose concentrations. The active transport of fructose, as demonstrated by the conversion of fructose in the isolated surviving guinea pig intestine, is enhanced by the presence of potassium ions (15.6 meq./liter). The rate of transport of fructose itself is unaffected by potassium. Using radioactive glucose and fructose, it is shown that the total amount of sugar transferred through the intestine as estimated by the radioactivity appearing in the serosal solution is approximately that calculated from chemical analyses. Potassium ions have no activating action on the transport of sugars such as sorbose, mannose, and D-glucosamine, but have a marked effect on galactose transport. The results support the conclusion that potassium ions do not influence active transport of glucose, fructose, and galactose by a change of intestinal permeability to these sugars, but do so by affecting a specific phase involved in the mechanism of active transport of sugars. The presence of L-glutamine stimulates active transport of glucose, whereas that of L-glutamate tends to diminish it.

Earlier studies (8, 10, 13) on sugar absorption from isolated surviving guinea pig small intestine, by the perfusion technique of Darlington and Quastel (8), have shown that this tissue brings about active absorption of glucose, fructose, and galactose, the absorption of fructose being accompanied by a transformation into glucose. An active respiratory system in the intestine is essential for optimal rates of these processes. After experimental (tourniquet) shock, the ability of the isolated guinea pig intestine to absorb glucose, or to transform fructose into glucose, is greatly reduced, a condition resembling that of anoxia being produced (13).

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The term intestinal absorption in these experiments implies passage of the absorbed substance from the lumen through the intestine into the solution bathing the serosal aspect of the intestine. It is known that two processes are involved in the absorption of sugar from the isolated intestine: an active process that is suppressed under anaerobic condition or by the presence of 2,4-dinitrophenol, and a passive diffusion process that is not affected by anaerobic condition but is markedly influenced by the concentration of sugar in the lumen. Recent work (15, 29) has shown that, in glucose transport through the guinea pig intestine, very little conversion to lactic acid takes place.

Attempts have been made to show that glucose absorption from the intestine is affected by changes in the salt balance. Thus, Althausen, Anderson, and Stockholm (1) demonstrated that decreased intestinal absorption of glucose in rats after adrenalectomy was restored to normal by addition of sodium chloride to the drinking water but this result has been questioned by Marrazzi (19). Clark and MacKay (5) concluded that a reduction of glucose absorption occurred when the sodium balance was disturbed. Magee and Sen (17, 18) have claimed that the presence of calcium is necessary for, or greatly facilitates, selective transport of glucose by surviving intestine, whilst Gellhorn and Skupa (12) have concluded that potassium and calcium ions affect the permeability of frog intestine to glucose. Gardner and Burget (11) found, working with closed loops of dog small intestine, that addition of potassium chloride (up to 0.1%) to the fluid bathing the intestine increased the rate of glucose absorption whilst addition of the same concentration of calcium chloride diminished the rate of absorption. Recently, Budolfsen (4), using segments of the small intestine and colon of white rats anaesthetized with urethane, has found that 0.45% potassium chloride causes a decrease of the rate of absorption of glucose from the ileum.

The perfusion technique of Darlington and Quastel (8) lends itself to a study of the effects of cationic changes in the mucosal and serosal solutions bathing isolated surviving guinea pig intestine on the rates of sugar absorption. This paper is concerned with a description of these effects.

Methods and Materials

Animals

Male guinea pigs weighing between 500 g. and 800 g. were used. They were fasted for 24 hours previous to an experiment but they were allowed to drink water.

Experimental Procedure

The experimental procedure adopted in this work is fully described by Darlington and Quastel (8) and need not be described again here. The principle of the perfusion technique which has been adopted consists of circulating through the lumen of a length of freshly excised guinea pig small intestine at 37° an oxygenated Ringer–bicarbonate solution containing the substances whose absorption by the intestine is being studied. This solution

is termed the mucosal solution. Bathing the intestine segment is an outer solution, also an oxygenated Ringer–bicarbonate solution, which circulates around the serosal surface of the intestine. This outer solution is termed the serosal solution. Both solutions are gassed continuously with 93% oxygen and 7% carbon dioxide. The apparatus is so arranged that conditions may easily be made anaerobic and substances may be added either to the mucosal or serosal solutions, or to both, at any time during the experiment.

Segments of guinea pig small intestine, 4 in. in length, are taken 12 and 16 in. from the pylorus.

The substance whose absorption is under study is present in the mucosal solution only. The rate of appearance of this substance in the serosal solution is measured by taking samples every 15 minutes from the serosal solution, analyzing them, and plotting the results.

It is usual to add 0.2% sorbose to the mucosal solution in addition to the substance under investigation. The absorption or diffusion of sorbose through guinea pig intestine is "passive." Its rate of appearance in the serosal solution serves as a useful check for any leak in the intestinal segment. If the rate of appearance of sorbose in the serosal solution is in the experimentally established "normal range" (i.e. between 2.0 and 7.0 μM./hour under our experimental conditions), it is considered that the segment is showing no undue permeability change and that the results for rates of absorption of the substance under investigation are valid. In experiments with fructose or sucrose sorbose is not added to the perfusion medium, as the rate of fructose appearance enables the detection of leaks in the intestine to be made.

It should be emphasized that "absorption", as studied by an *in vitro* technique such as that adopted here, refers to the transfer of substances through the entire intestinal wall. Results already obtained by this technique show close correspondence with those obtained by *in vivo* procedures and have been confirmed by workers using other *in vitro* methods.

Since the object of the present work is to assess the effects of cations on sugar absorption, the constitutions of the Ringer–bicarbonate solutions bathing the intestine have been varied. These changes are noted in the various tables of results given below.

The presence of calcium ions causes excessive motility of the intestinal segment, which may interfere with the efficiency of circulation through the lumen. For this reason calcium is usually omitted from the Ringer–bicarbonate medium. Its omission had not been found markedly to affect the rate of absorption of glucose (8). Usually its presence, at 2.6 meq./liter brings about a decrease of approximately 30% of the rate of absorption of glucose found in the absence of added calcium ions.

Composition of Ringer–Bicarbonate (Calcium-free) Medium

The Ringer–bicarbonate solution was prepared as described by Umbreit *et al.* (26), calcium ions, however, being omitted from the medium. The composition is as follows: NaCl = 0.714% (0.122 *M*); $NaHCO_3$ = 0.208% (0.025 *M*); KCl = 0.036% (0.0048 *M*); KH_2PO_4 = 0.017% (0.0012 *M*); $MgSO_4 \cdot 7H_2O$ = 0.030% (0.0012 *M*).

Analytical Methods

Sorbose and fructose have been measured, using the Beckmann spectrophotometer at 525 mμ by the method of Roe (23). Total reducing substances were measured spectrophotometrically (at 520 mμ) by the methods of Somogyi (25) and Nelson (21). Estimates of sorbose and fructose determined in the presence of varying quantities of glucose were satisfactory. As either fructose or sorbose was usually present in the perfusion medium the amount of glucose was determined by difference. Fridhandler and Quastel (10) using the notatin manometric technique have demonstrated the validity of this procedure.

Use of Radioactive Substances

Experiments were carried out in the standard perfusion apparatus, samples being taken for radioactive counting and for chromatography. The samples taken for counting were plated on aluminum disks and counted in a Geiger–Müller windowless flow counter. Results were corrected for self-absorption. When necessary for chromatography the volume of the whole serosal solution at the end of an experiment was reduced by lyophilization. Paper chromatograms were scanned for radioactive spots whose positions were compared with those of known standards.

Chromatography

Descending paper chromatography (Whatman No. 1 paper) was used at room temperature, to detect sugars in the mucosal and serosal solutions. The solvent used was *n*-butanol:acetic acid:water (4:1:5) (17, 22). A benzidine and trichloracetic acid spray was used to develop the sugar spots (3). In ascending paper chromatograms *n*-propanol:ethyl acetate:water 7:1:2 (2) was used as solvent and the spray was benzidine – trichloracetic acid. The chromatograms were dried after spraying and heated for 5–10 minutes at 110° C.

Results of Sampling

Samples of the serosal solution were taken at 15-minute intervals over a period of 75 minutes, 2 ml. were taken for assay of total reducing sugar, 1 ml. for assay of keto sugar. The circulating volume was kept constant by the addition of corresponding volumes of Ringer–bicarbonate solution. The results of the analyses of aliquots were compared with standard curves and converted to the corresponding values for the entire volume of the serosal solution. Corrections were made for concentration changes brought about by the maintenance of a constant circulating volume. Results could be expressed in the form of curves, of which a typical curve for glucose absorption is shown in Fig. 1. Results are usually expressed as rates of transport of the test substance. For this purpose, the time interval taken is the period from 15 to 75 minutes and final results are given as μM. per hour. Experiments were carried out at least in triplicate and generally results could be reproduced within a relatively small range ($\pm 6\%$) of variation.

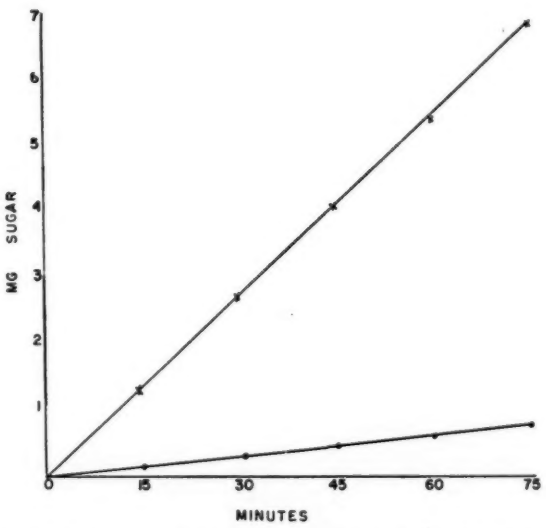


FIG. 1. Rate of glucose transport by isolated surviving guinea pig intestine.
Ordinate: mg. sugar transported into serosal solution.
Abscissa: minutes from beginning of perfusion of isolated intestine.
Gas: 93% O_2 plus 7% CO_2; Ringer–bicarbonate (calcium-free) solution; K^+=6 meq./liter.
×=Glucose 250 mg.% (initially in mucosal solution). ●=Sorbose 250 mg.% (initially in mucosal solution).

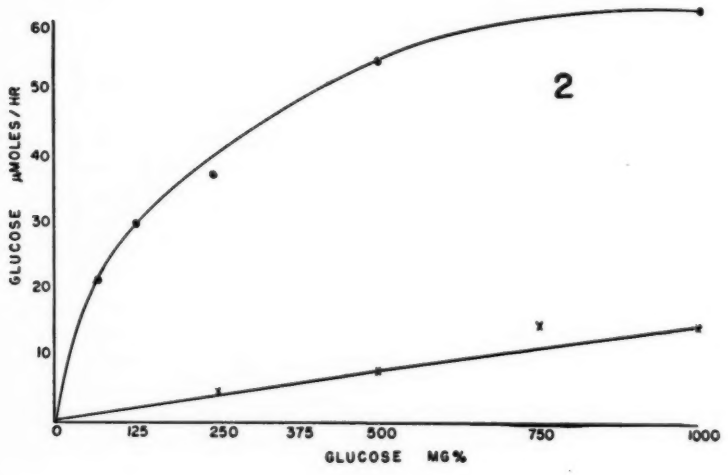
Results

Endogenous Sugar Transport

It has been shown already (8) that when the mucosal solution contains no sugar other than sorbose there appears in the serosal solution small quantities of glucose (10). Its rate of appearance is about 10 μM. per hour. Experiments show that this rate of "endogenous" glucose appearance is obtained when intestines of well fed guinea pigs are used. When the animals are starved for 24 hours before the intestine is removed the rate of "endogenous" glucose appearance in the serosal fluid is only about 2 μM. per hour. The rates of endogenous glucose transport are reduced very considerably under anaerobic conditions, indicating that "endogenous" glucose is actively transported. Only minute quantities of glucose (<0.5 μM./hour) are found in the mucosal solution, under the conditions when larger quantities appear in the serosal solution. This points to the existence of a unidirectional transport system for the sugar in the isolated guinea pig intestine (see also Ref. 9). In experiments described below, guinea pigs fasted for 24 hours have been used.

Variation of the Rate of Glucose Transport with Glucose Concentration

Experiments have been carried out to investigate the relation between glucose transport through the isolated surviving guinea pig intestine and glucose concentration in the mucosal solution.



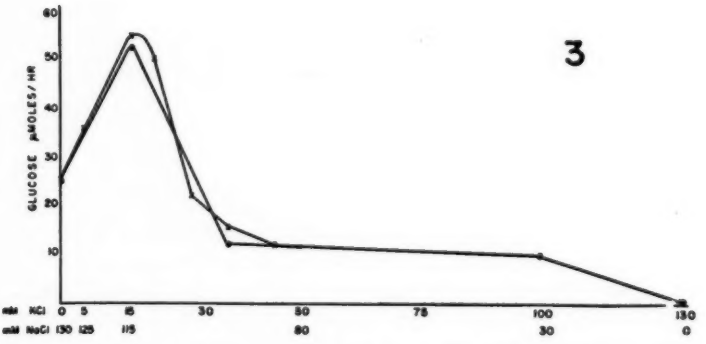


FIG. 2. Rate of glucose transport in relation to initial mucosal concentration of glucose.
Ordinate: μM./hour glucose transported into serosal solution.
Abscissa: Initial concentration of glucose in mucosal solution.
Gas: 93% O_2 plus 7% CO_2 or 93% N_2 plus 7% CO_2; Ringer–bicarbonate (calcium-free) solution; $K^+ = 6$ meq./liter.
● = Glucose, aerobic. × = Glucose, anaerobic.

FIG. 3. Rate of transport of glucose in relation to potassium ion and sodium ion content of perfusing Ringer solution.
Ordinate: μM./hour glucose transported into serosal solution.
Abscissa: Concentrations of K^+ and Na^+ given as millimoles KCl and NaCl present in perfusing Ringer–bicarbonate (calcium-free) solution. These concentrations are in addition to 1.2 millimolar concentration of potassium phosphate present.
× = Glucose, K^+ variable; Na^+ constant = 122 meq./liter. ● = Glucose, K^+ variable and Na^+ variable, but $K^+ + Na^+$ = constant (128 meq./liter).

Representative results are shown in Fig. 2. They show that, with increasing glucose concentrations, under aerobic conditions, a maximum rate of glucose appearance in the serosal fluid is obtained. The relation between the rate of absorption of glucose and the initial glucose concentration resembles a relation of the Michaelis–Menten type. Under anaerobic conditions (when the Ringer–bicarbonate solution is gassed with 93% nitrogen and 7% carbon dioxide), the rate of glucose transport is much smaller than that obtained under aerobic conditions and increases proportionally with increase of glucose concentration in the mucosal solution. On plotting reciprocals of aerobic rates of transport of glucose against reciprocals of mucosal concentrations of glucose, as in a Lineweaver–Burke analysis, a straight line is obtained giving an *apparent* $K_m = 7 \times 10^{-3}$ *M* (half saturation concentration). It is of interest to note that Fisher and Parsons (20), in their studies on the effects of glucose concentration on rat intestinal absorption, found that the half saturation concentration of glucose was 9×10^{-3} *M*. This value of an apparent K_m for glucose transport makes it unlikely that hexokinase, if it is involved in the phenomenon of active glucose absorption, is rate limiting, as known values (6, 7, 28, 24, 14) for the K_m of hexokinase of animal tissues for glucose are much smaller than 7×10^{-3} *M*. In fact it is more reminiscent of the value of the Michaelis constants for phosphatases (e.g. glucose-6-phosphatase of liver, $K_m = 2 \times 10^{-3}$ (6)).

Effects of Variation of Concentration of Potassium Chloride on Rates of Absorption of Glucose and Sorbose

Results given in Table I demonstrate the changes that take place in the rates of absorption of glucose, under aerobic conditions, when the potassium chloride concentration is altered in a calcium-free Ringer solution. In all experiments in which the effects of potassium ions on glucose absorption are studied, two

TABLE I

AEROBIC ABSORPTION OF GLUCOSE IN PRESENCE OF VARYING KCl CONCENTRATIONS

Mucosal solution: Ringer–bicarbonate (calcium-free) solution. Glucose 250 mg.%. Sorbose 200 mg.%.
Serosal solution: Ringer–bicarbonate (calcium-free) solution.
Gas: 93% O_2 plus 7% CO_2. Time, 75 min. Temp., 38° C.

No. expts.	Concn. of KCl in Ringer soln. (apart from 1.2 m*M* K^+ present as potassium phosphate), m*M*	Serosal soln., rate of appearance, μM./hour, with standard deviations	
		Glucose	Sorbose
4	0.0	26.0 ± 2.9	6.0 ± 0.8
8	4.8	36.4 ± 3.7	2.7 ± 0.5
4	9.6	46.0 ± 3.8	6.0 ± 1.4
11	14.4	55.0 ± 5.9	4.6 ± 2.6
3	19.2	51.6 ± 6.4	5.1 ± 1.4
4	26.4	21.5 ± 3.3	6.8 ± 1.2
3	33.6	15.7 ± 3.0	4.4 ± 1.0
3	43.2	15.6 ± 3.2	4.6 ± 1.1

intestinal segments from the same animal are used. One segment is perfused with a normal Ringer–bicarbonate solution containing glucose and 6 meq./liter K^+. This serves as a control for the other segment that is investigated for potassium ion effects. The normal rate of glucose absorption (i.e. with $K^+ =$ 6 meq./liter and glucose = 14 m*M*), is about 36 μM./hour.

Increase of the concentration of potassium chloride in the Ringer–bicarbonate solution leads to a marked increase of the rate of glucose absorption. The optimal rate of glucose absorption occurs in the presence of 14.4 m*M* potassium chloride (i.e. total $K^+ = 15.6$ meq./liter). The lowest rates of glucose absorption in calcium-free Ringer solution occur either in the entire absence of potassium ions, when the rate is approximately 20 μM./hour, or in the presence of concentrations of potassium ions exceeding 35 meq./liter. In all experiments in which the potassium chloride content of the Ringer solution exceeds 5 m*M* increased peristaltic movements of the intestinal segment are noticed. The optimal rate of glucose absorption is approximately 55 μM./hour (see also Fig. 3).

Diffusion of sorbose is not appreciably affected by altered potassium ion concentrations (Table I) indicating no non-specific alteration of permeability of the intestine.

Potassium ions do not stimulate glucose absorption under anaerobic conditions. The average rate of anaerobic absorption of glucose in the absence of potassium ions is approximately 4 μM./hour and this is scarcely affected by increase of potassium ion concentration to 15.6 meq./liter (Table II). Thus it would appear that the stimulating action of potassium ions is concerned only with the process of active absorption of the sugar.

On comparing the effects of increased K^+ concentration on glucose absorption at different glucose concentrations it is seen that the optimal K^+ effect is exerted when the mucosal glucose concentration is about 250 mg.% (14 m*M*). Below this concentration of glucose little stimulant action of potassium ion is seen, whereas above it the rate of glucose transport increases (even in the

TABLE II

ANAEROBIC ABSORPTION OF GLUCOSE IN PRESENCE OF VARYING KCl CONCENTRATIONS

Mucosal solution: Ringer–bicarbonate (calcium-free) solution. Glucose 250 mg.%. Sorbose 200 mg.%.
Serosal solution: Ringer–bicarbonate (calcium-free) solution.
Gas: 93% N_2 plus 7% CO_2. Time, 75 min. Temp. 38° C.

No. expts.	Concn. of KCl in Ringer soln. (apart from 1.2 m*M* K^+ present as potassium phosphate), m*M*	Serosal solution, rate of appearance, μM./hour	
		Glucose	Sorbose
3	0.0	4.5	7.0
7	4.8	4.4	4.8
3	9.6	3.0	6.3
8	14.4	3.1	5.3

absence of added K^+) to the maximum value found with the high potassium ion (15.6 meq./liter) concentrations. Representative results are shown in Table III. The effect of increase of potassium ion concentration is to accelerate the rate of transport found with relatively low concentrations of glucose to the maximum value obtained with high concentrations of the sugar. Perhaps the phenomenon of potassium stimulation can be interpreted as due to an acceleration of the rate of conversion of glucose to hexose phosphate prior to hydrolysis by intestinal phosphatase.

Further experiments showed that the stimulating action of potassium chloride is given by an equivalent concentration of potassium nitrate, so that the effect is clearly attributable to the potassium ions. Nevertheless, the presence of nitrate ions seems to cause some diminution of the rate of aerobic glucose absorption.

TABLE III

AEROBIC ABSORPTION OF GLUCOSE BY ISOLATED SURVIVING GUINEA PIG INTESTINE AT DIFFERENT GLUCOSE AND POTASSIUM ION CONCENTRATIONS

Mucosal solution: Ringer–bicarbonate (calcium-free) solution. Sorbose 200 mg.%. Glucose, as stated below. K^+ either 6 meq./liter or 15.6 meq./liter.
Serosal solution: Ringer–bicarbonate (calcium-free) solution. K^+ either 6 meq./liter or 15.6 meq./liter.
Gas: 93% O_2 plus 7% CO_2. Time, 75 min. Temp., 38° C.

Expts.	Glucose concn., *M*	Serosal solution, rate of appearance of glucose, μM./hr., in presence of: 6 meq./l. K^+	15.6 meq./l. K^+
3	0.0035	21	23
5	0.007	31	32
5	0.014	36	55
3	0.021	48	50.5
3	0.028	54	53

Effects of Sodium Ions on Glucose Absorption by Isolated Surviving Guinea Pig Intestine

In the experiments, the results of which are recorded in Tables I, II, III, the sodium chloride content of the Ringer solution is kept constant at 122 meq./liter. If the relative ionic concentrations of potassium and sodium are changed so that the sum of KCl + NaCl is constant (at 126.8 m*M*) results are obtained which are shown in Fig. 3. They indicate that, in the absence of sodium ions, when the sodium chloride has been replaced by potassium chloride, active glucose absorption by the isolated surviving intestine is abolished. Values of the order of 3 μM./hour or less are obtained. The stimulating action of 15.6 meq./liter potassium ions is approximately the same whether the sodium ion concentration is 112 meq./liter or 122 meq./liter.

TABLE IV

EFFECTS OF POTASSIUM IONS IN THE MUCOSAL OR SEROSAL SOLUTIONS ON THE RATE OF GLUCOSE ABSORPTION BY ISOLATED GUINEA PIG INTESTINE

Mucosal solution: Ringer–bicarbonate (calcium-free) solution. Glucose 250 mg.%. Sorbose 200 mg.%.
Serosal solution: Ringer–bicarbonate (calcium-free) solution.
Gas: 93% O_2 plus 7% CO_2. Time, 75 min. Temp., 38° C.

meq./l. K^+ present in Ringer solution		Serosal solution, rate of appearance of glucose, μM./hr.
Mucosal soln.	Serosal soln.	
0	0	20
1.2	1.2	26
6	1.2	22
15.6	1.2	28
1.2	6	20
1.2	15.6	44
6	6	35
15.6	15.6	55

It is evident that sodium ions must be present for glucose absorption to occur and that, also under isotonic conditions, potassium ions exert a stimulation of the rate of glucose transport in the guinea pig intestine.

When experiments are carried out with different concentrations of potassium ions in the mucosal and serosal solutions, it is observed that optimal stimulating action of potassium occurs only when the potassium ion concentration is the same in both mucosal and serosal solutions. Representative results are shown in Table IV. It is evident that increase of potassium ion concentration in the mucosal solution only has no appreciable effect on glucose transport and that maximal rates of glucose absorption occur when there is no concentration gradient of potassium ions across the intestinal wall.

Effects of Magnesium Ions on Glucose Absorption by Isolated Surviving Guinea Pig Intestine

Magnesium ions are normally present in the Ringer–bicarbonate solution, used in these experiments, at a concentration of 1.2 meq./liter. Experiments were carried out to see if variation of magnesium ion concentration affected glucose transport. Some typical results are presented in Table V. They show that, in the absence of added potassium chloride to the Ringer solution, addition of magnesium sulphate to 20 m*M* had no marked effect on glucose transport. Moreover when potassium chloride was present at 4.8 m*M* concentration in the Ringer solution the omission of magnesium sulphate had only a slight diminishing effect on glucose transport. In the presence of 15.6 meq./liter potassium ions the omission of magnesium ions greatly decreases the active transport of glucose. The stimulated rate of 55 μM./hour is reduced to 20 μM./hour, a value which is even below that obtained in the absence of added potassium ions. Increase of magnesium ion concentration

TABLE V

EFFECTS OF MAGNESIUM AND AMMONIUM IONS ON THE ABSORPTION OF GLUCOSE BY ISOLATED SURVIVING GUINEA PIG INTESTINE

Mucosal solution: Ringer–bicarbonate (calcium-free) solution. Glucose 250 mg.%. Sorbose 200 mg.%. Varying salts as stated below.
Serosal solution: Ringer–bicarbonate (calcium-free) solution. Varying salts as stated below.
Gas: 93% O_2 plus 7% CO_2. Time, 75 min. Temp., 38° C.

meq./l. K^+ present in Ringer soln.	m*M* $MgSO_4$ in Ringer soln.	m*M* NH_4Cl in Ringer soln.	Serosal solution, rate of appearance of glucose, μM./hr.
1.2	0	0	30
1.2	1.2	0	26
1.2	20	0	36.5
6.0	0	0	26
6.0	1.2	0	35
6.0	12	0	44
6.0	20	0	40
15.6	0	0	20
15.6	1.2	0	55
15.6	12	0	40
15.6	20	0	41
1.2	1.2	5	22
1.2	1.2	15	9.3
6.0	1.2	15	23.5
15.6	1.2	15	31

above that normally present in Ringer solution (i.e. 1.2 meq./liter) does not increase the glucose absorption rate obtained with 15.6 m*M* K^+ but tends to diminish it.

The necessity for magnesium ions for the stimulated rate of glucose absorption secured by potassium indicates an important role of magnesium in the process of intestinal glucose transport. The effect is consistent with the known activating effects of magnesium ions on both phosphorylation and phosphatase activities (i.e. on hexokinase (16) or intestinal phosphatase (20)).

Effects of Ammonium Ions on Glucose Absorption by Isolated Surviving Guinea Pig Intestine

Results recorded in Table V show that the presence of ammonium ions brings about a diminution of the rate of glucose absorption by isolated surviving guinea pig intestine. Such a result might be expected if ammonium ions interfere with the metabolic activity of intestinal tissue in the presence of glucose. It is of interest, in this connection, to note that anaerobic glycolysis of brain cortex is diminished by ammonium ions (Weil-Malherbe (27)).

Effects of Rubidium Ions on Glucose Absorption by Isolated Surviving Guinea Pig

Rubidium ions resemble potassium ions in bringing about a stimulation of glucose transport in isolated surviving guinea pig intestine. For example, a glucose transport of 26 μM./hour in the presence of 1.2 meq./liter K^+ was

increased to 35 μM./hour by the addition of 5 m*M* rubidium chloride and to 52 μM./hour by the addition of 15 m*M* rubidium chloride. The addition of cesium chloride (5 m*M* and 15 m*M*) had no activating effects on the rate of glucose transport.

Effects of Potassium Ions on Fructose Absorption by Isolated Surviving Guinea Pig Intestine

As has been shown previously (8, 10) fructose is converted to glucose on its passage through the isolated surviving guinea pig intestine. It was demonstrated (10) that, with 28 m*M* fructose, less than one-half of the sugar appearing in the serosal solution is fructose, the rest is glucose. With higher concentrations of fructose, the rate of transformation into glucose is not appreciably increased but there is an increased rate of diffusion of fructose into the serosal solution. We have extended these experiments using fructose concentrations smaller than 28 m*M*. It is found that with 7 m*M* fructose over 80% of the sugar transported is glucose, and the proportion of glucose to fructose decreases with increase of fructose concentration in the lumen. Typical results are shown in Table VI, where it will be seen that increase of potassium ion concentration (in the mucosal and serosal solutions) to 15.6 meq./liter greatly increases the proportion of glucose appearing in the serosal fluid. At a mucosal concentration of 7 m*M* fructose, the amount of glucose

TABLE VI

EFFECTS OF POTASSIUM IONS ON FRUCTOSE ABSORPTION BY ISOLATED SURVIVING INTESTINE

Mucosal solution: Ringer-bicarbonate (calcium-free) solution. Fructose concentration as stated below.
Serosal solution: Ringer–bicarbonate (calcium-free) solution.
Gas: 93% O_2 plus 7% CO_2. Time, 75 min. Temp., 38° C.

No. expts.	Concn. of fructose in mucosal soln., *M*	Concn. (meq./l.) of K^+ in both mucosal and serosal soln.	Serosal solution, rate of appearance, μM./hr.	
			Glucose	Fructose
3	0.007	6.0	8.3	1.9
4	0.007	15.6	19.2	2.1
3	0.014	6.0	12.3	5.3
4	0.014	15.6	22.0	5.6
4	0.028	6.0	15.2	15.7
3	0.028	15.6	19.2	14.6

appearing in the serosal solution is more than double that appearing when only 6 meq./liter K^+ are present. The increase of potassium ion concentration has no effect on the rate of appearance of fructose, but stimulates the total transport of sugar, its stimulating effect being greatest with low concentrations of fructose. Thus, as with glucose, the effect of increased K^+ is to accelerate the rate of active transport to the maximum value.

TABLE VII

ABSORPTION OF RADIOACTIVE SUGARS BY ISOLATED SURVIVING GUINEA PIG INTESTINE

Mucosal solution: Ringer–bicarbonate (calcium-free) solution. Glucose present = 125 mg. Fructose present = 130 mg. Sorbose 200 mg.% (present only in the glucose transport expts.).
Serosal solution: Ringer–bicarbonate (calcium-free) solution.
Gas: 93% O_2 plus 7% CO_2. Time, 75 min. Temp. 38° C.

	Serosal solution, appearance in 75 min.	
Mucosal solution, total activity	Total activity (corrected)	Reducing sugars (found chemically)
Fructose 1,950,000 c.p.m. i.e. c.p.m./μg. fructose = 15	72,000 c.p.m. = 4800 μg. sugar	4450 μg.
Glucose 2,117,500 c.p.m. i.e. c.p.m./μg. glucose = 17	128,000 c.p.m. = 7530 μg. glucose	8400 μg. glucose

Absorption of Radioactive Sugars by Isolated Surviving Guinea Pig Intestine

It has been shown by Kiyasu and Chaikoff (15) that in the guinea pig, fed with fructose-C^{14}, about 30% of the C^{14} in portal plasma was present as fructose, 10% as lactate, and 60% as glucose, confirming earlier observations (8) on the conversion of fructose into glucose in the guinea pig intestine. Experiments were carried out on the absorption of fructose-U-C^{14} and glucose-U-C^{14} by isolated surviving guinea pig intestine,* and some typical results are given in Table VII. They indicate that the total amount of sugar appearing in the serosal solution as estimated by the radioactivity is approximately that calculated from chemical analysis. The approximate agreement indicates that the fructose and glucose appearing in the outer solution accounts for the radioactivity found there.

Chromatograms were developed from both mucosal and serosal solutions, and radioactivity was found in the spots corresponding to the positions of glucose and fructose. No radioactivity was detected elsewhere in the chromatograms. If radioactive lactic acid was present, it was in too small amount to be detected by present techniques.

Effects of Potassium Ions on the Absorption of Sorbose, Mannose, Galactose, and D-*Glucosamine by Isolated Surviving Guinea Pig Intestine*

The stimulating action of added potassium ions is as marked on the absorption of galactose as it is on that of glucose. On the other hand, no effect of increased potassium ion concentration was seen on the absorption of sorbose, mannose, or D-glucosamine. Typical results are shown in Table VIII. No evidence of active transport of these three sugars has been found (8, 10). The results give strong support to the conclusion that potassium ions do not

*Fructose-U-C^{14} and glucose-U-C^{14} indicate uniformly labelled substances.

TABLE VIII

EFFECTS OF POTASSIUM IONS ON THE ABSORPTION OF SORBOSE, MANNOSE, GALACTOSE, AND D-GLUCOSAMINE BY ISOLATED SURVIVING GUINEA PIG INTESTINE

Mucosal solution: Ringer–bicarbonate (calcium-free) solution. Sorbose 200 mg.%. Other sugars as stated below.
Serosal solution: Ringer–bicarbonate (calcium-free) solution.
Gas: 93% O_2 plus 7% CO_2. Time, 75 min. Temp., 38° C.

Mucosal solution, sugar present, mg.%	Concn. of K^+ (meq./l.) in mucosal and serosal solutions	Serosal solution, rate of appearance in μM./hr.
Sorbose, 200	6.0	3
	15.6	4
Mannose, 250	6.0	7.2
	15.6	8.0
Galactose, 250	6.0	36.5
	15.6	62.2
D-Glucosamine, 250	6.0	5.4
	15.6	5.6

influence the active transport of glucose, fructose, or galactose by changing the permeability of the intestinal tissues to sugars, but that potassium ions act by affecting a specific phase involved in the mechanism of active transport of sugars.

Effects of L-*Glutamine and Sodium* L-*Glutamate on Glucose Absorption by Isolated Surviving Guinea Pig Intestine*

The addition of L-glutamine exercises a stimulating effect on the rate of glucose absorption by isolated guinea pig intestine and this stimulation is still to be seen in presence of 15.6 meq./liter potassium ions. The presence of sodium L-glutamate, however, tends to diminish the rate of glucose absorption by the intestine. Representative results are given in Table IX.

TABLE IX

EFFECTS OF L-GLUTAMINE AND SODIUM L-GLUTAMATE ON GLUCOSE ABSORPTION BY ISOLATED SURVIVING GUINEA PIG INTESTINE

Mucosal solution: Ringer–bicarbonate (calcium-free) solution. Glucose = 250 mg.%; sorbose = 200 mg.%.
Serosal solution: Ringer–bicarbonate (calcium-free) solution.
Gas: 93% O_2 plus 7% CO_2. Time, 75 min. Temp., 38° C.

No. expts.	K^+ concn. in mucosal and serosal soln.	Concn. of amino acid in mucosal soln., m*M*	Serosal solution, rate of appearance, μM./hr. of:	
			Glucose	Sorbose
3	6.0	Nil	36.0	3
4	6.0	L-Glutamine, 10	42.5	2.5
4	6.0	L-Glutamate, 10	29.7	3
3	15.6	Nil	55.0	3
4	15.6	L-Glutamine, 10	65.1	3.2
3	15.6	L-Glutamate, 10	40.0	4.6

The reasons for these effects are at present obscure but it seems possible that the phenomena may be linked with adenosine triphosphate formation or breakdown during glutamate–glutamine interconversion.

Discussion

The facts presented in this paper show that the process of active absorption of sugars (glucose, galactose, and fructose) by the isolated surviving guinea pig intestine is markedly affected by the cation concentrations present in the salt solutions bathing the intestine. The most marked effect is that presented by potassium ions. The maximum rate of glucose transfer is about 55 μM. per hour (under the given experimental conditions), this rate being secured by the presence of 15.6 meq./liter K^+ and 14 mM glucose. With a normal concentration of $K^+ = 6$ meq./liter the rate of transport of glucose (for 14 mM glucose) is 36 μM. per hour. The higher rate of glucose transport may also be obtained by concentrations of glucose exceeding 28 mM in presence of 6 meq./liter K^+. The effect of the potassium ions is to bring about an acceleration of the rate at which the maximum velocity of transfer of glucose is obtained.

Sodium ions seem to be essential for the process of active transport, as their omission and replacement by potassium ions abolishes all active transport of glucose. Small variations of sodium ions from the normal concentration of 122 meq./liter produce, however, no differences in the rates of glucose transport. Magnesium ions also are necessary for active glucose transport, especially for securing the stimulating action of 15.6 meq./liter potassium ions.

The stimulation of active glucose transport in the intestine found with potassium ions may also be observed with rubidium ions, but not with cesium ions.

Ammonium ions depress the rate of active glucose transport in the guinea pig intestine. It is possible that this effect is linked with glutamate–glutamine metabolism in the intestine. This suggestion finds support in our observations that whereas the presence of L-glutamine stimulates active transport of glucose, that of L-glutamate tends to diminish it.

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EFFECTS OF METABOLIC INHIBITORS ON POTASSIUM-STIMULATED GLUCOSE ABSORPTION BY ISOLATED SURVIVING GUINEA PIG INTESTINE[1]

E. RIKLIS[2] AND J. H. QUASTEL

Abstract

2,4-Dinitrophenol, at low concentrations, inhibits potassium-stimulated active transport of glucose by the isolated surviving guinea pig intestine to a greater extent than the unstimulated glucose transport. The potassium stimulation is abolished in the presence of 0.04 m*M* 2,4-dinitrophenol. Potassium stimulation of the active transport of glucose and galactose in the isolated guinea pig intestine is inhibited by phlorizin at low concentrations (0.01 m*M*) which have little or no effect on the unstimulated sugar transport. The presence of phlorizin has little or no effect on active fructose absorption, as shown by the combined transport of fructose and glucose derived from the fructose. In the presence of 15.6 meq./liter K^+ phlorizin exerts a small depression of the active transport of fructose. Potassium stimulation of the active transport of glucose in the isolated guinea pig intestine is inhibited by the narcotic luminal at low concentrations (2 m*M*). Luminal (10 m*M*) abolishes the potassium stimulation. Sodium malonate, at the concentration 2 m*M*, which exerts no inhibition of active glucose transport in isolated surviving guinea pig intestine, brings about over 40% inhibition of glucose transport when this is stimulated by potassium ions. Choline, at 0.5 m*M*, suppresses potassium stimulation of the active glucose transport in the isolated surviving guinea pig intestine. It is suggested that an enzymatic mechanism exists, associated with intestinal membranes, that controls sugar transport and that phosphorylations, either directly or indirectly, are connected with it.

Introduction

It has been shown in the preceding paper (21) that an increase to 15.6 meq./liter of potassium ion concentration of the Ringer–bicarbonate solution bathing isolated surviving guinea pig intestine brings about a marked increase in the rate of active absorption of glucose and galactose from the intestine when these sugars are present at a concentration of 14 m*M* and in the rate of transport of glucose derived from fructose. Experiments now show that a number of metabolic inhibitors affect potassium-stimulated active glucose absorption by the intestine to a much greater extent than the unstimulated active glucose absorption by the intestine. This paper is concerned with a description of these phenomena.

Methods and Materials

The experimental procedure adopted is that fully described by Darlington and Quastel (5) and used in the work described in our preceding paper (21). The materials and analytical methods used are also described in our earlier paper (21). Metabolic inhibitors are added to the mucosal solution at the same time as the sugars.

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Potassium-Stimulated Active Transport

The perfusion medium used in these experiments is a Ringer–bicarbonate (calcium-free) medium whose complete composition is given in our earlier paper (21). The normal concentration of K^+ in this medium is 6 meq./liter. When the concentration of K^+ is increased to 15.6 meq./liter the resulting higher rate of active transport of certain sugars is referred to as the potassium-stimulated active transport.

Results

Effects of 2,4-Dinitrophenol (DNP) on Glucose Absorption by the Isolated Surviving Guinea Pig Intestine

It is known that addition of 10^{-4} *M* DNP to the Ringer–bicarbonate solution bathing the isolated guinea pig intestine abolishes active absorption of glucose (5) and the conversion of fructose into glucose (7) without interference with the transport of fructose itself.

Further experiments, typical results of which are shown in Table I, demonstrate that DNP at low concentrations affects the potassium-stimulated active transport of glucose to a greater extent than the unstimulated active transport of glucose. In fact, the effect of low concentrations of DNP (e.g. 4×10^{-5} *M*) is to abolish, apparently, the stimulating action of potassium. The lowest rate of transport of glucose in the presence of DNP was found to be 5 μM./hour in the presence of both 6 meq./liter K^+ and 15.6 meq./liter K^+; this rate of transport is approximately the passive diffusion rate which is found under anaerobic conditions.

It is evident that DNP acts most markedly on a specific phase of the mechanism of glucose transport in the intestine, a phase that is stimulated by the presence of potassium ions.

TABLE I

EFFECTS OF 2,4-DINITROPHENOL (DNP) ON GLUCOSE ABSORPTION BY ISOLATED SURVIVING GUINEA PIG INTESTINE

Mucosal solution: Ringer–bicarbonate (calcium-free) solution. Glucose = 250 mg.%. Sorbose 200 mg.%.
Serosal solution: Ringer–bicarbonate (calcium-free) solution.
Gas: 93% O_2 plus 7% CO_2. Time, 75 min. Temp., 38° C.

Concn. of K^+ (meq./l.) in mucosal and serosal solution	Concn. of DNP in mucosal and serosal solution, m*M*	Serosal solution, rate of appearance, μM./hr.		% inhibition of glucose absorption
		Glucose	Sorbose	
6.0	Nil	36	3	—
6.0	0.02	34	2.6	5
6.0	0.04	26	4.3	28
6.0	0.1	5.9	2.1	83
6.0	0.5	4.9	2.4	87
15.6	Nil	55	3	—
15.6	0.02	41	3.4	25
15.6	0.04	26	4.4	53
15.6	0.1	16.3	4.5	73
15.6	0.2	6	3.6	89
15.6	0.4	6	4	89

TABLE II

EFFECTS OF PHLORIZIN ON SUGAR ABSORPTION FROM ISOLATED SURVIVING GUINEA PIG INTESTINE

Mucosal solution: Ringer–bicarbonate (calcium-free) solution. Sugar concentrations as stated below. Sorbose = 200 mg.%.
Serosal solution: Ringer–bicarbonate (calcium-free) solution.
Gas: 93% O_2 plus 7% CO_2. Time, 75 min. Temp., 38° C.

Sugar used	Concn. of K^+ (meq./l.) in mucosal and serosal solution	Concn. of phlorizin in mucosal and serosal solution, m*M*	Serosal solution, rate of appearance, μM./hr.		% inhibition of sugar transport
			Sugar	Sorbose	
Glucose, 250 mg.%	6.0	Nil	35	3	—
	6.0	0.01	35	3.8	0
	6.0	0.1	18	2.5	48
	6.0	1	8.1	3.5	77
	15.6	Nil	55	3	—
	15.6	0.01	35	3	36
	15.6	0.1	15	4.3	73
	15.6	1	8	3.5	86
Galactose, 250 mg.%	6.0	Nil	35	3	—
	6.0	0.01	37	4	0
	6.0	0.1	8.3	2.5	77
	15.6	Nil	55	3	—
	15.6	0.01	38.3	2.5	31
	15.6	0.1	14	3.9	75
Fructose, 250 mg.%			Glucose	Fructose	
	6.0	Nil	12	4	—
	6.0	0.1	11	6	0
	6.0	0.5	12	5	0
	15.6	Nil	18	5	—
	15.6	0.1	13	7	15
	15.6	0.5	14	4	22

Effects of Phlorizin on Sugar Absorption from the Isolated Surviving Guinea Pig Intestine

The results of recent experiments (7) with isolated surviving guinea pig intestine have confirmed earlier observations on the inhibitory effects of phlorizin on glucose absorption by intestine both *in vitro* and *in vivo* (6, 14, 17) and on the lack of inhibition by phlorizin on fructose absorption *in vivo* (1).

Experiments, the results of which are recorded in Table II, show that phlorizin at low concentrations (10^{-5} *M*) has a considerably larger inhibitory effect on potassium-stimulated absorption of both glucose and galactose than it has on the unstimulated active absorption rates of either of these sugars. It is noteworthy that the sensitivity of the rate of galactose absorption to phlorizin is at least equal to that of glucose absorption. Sorbose absorption is unaffected.

The absorption of fructose (calculated as the sum of fructose and glucose appearing in the serosal solution) is unaffected by concentrations of phlorizin (e.g. 10^{-4} *M*) that markedly inhibit the unstimulated absorption rates of

glucose and galactose. Some retarding effect of phlorizin is noticeable, however, on the rate of fructose absorption (calculated as transferred fructose and glucose) when this has been stimulated by the presence of 15.6 meq./liter K^+. The effect, however, is smaller than that observed, under equivalent experimental conditions, with glucose and galactose transport.

As with 2,4-dinitrophenol, phlorizin apparently exerts its largest inhibitory effect on that phase of the mechanism of the active transport of sugar which is stimulated by the presence of potassium ions.

Effects of Luminal on the Absorption of Glucose by Isolated Surviving Guinea Pig Intestine

Ghosh and Quastel (9) have shown that narcotics such as the barbiturates exert highly inhibitory effects on potassium-stimulated respiration of brain cortex slices in the presence of glucose or of pyruvate, the effect of the narcotic at low concentrations being to eliminate the potassium stimulation. Thus a narcotic such as pentothal at a concentration of 0.1 m*M* which may exert little or no inhibition of the rate of respiration of rat brain cortex in the presence of glucose exerts a complete suppression of the effect of potassium in stimulating the brain respiration. This stimulation of the normal brain respiration *in vitro* may amount to an increase of about 100%. The effect of a

TABLE III

EFFECTS OF LUMINAL AND OF SODIUM MALONATE ON GLUCOSE ABSORPTION BY ISOLATED SURVIVING GUINEA PIG INTESTINE

Mucosal solution: Ringer–bicarbonate (calcium-free) solution. Glucose = 250 mg.%. Sorbose = 200 mg.%.
Serosal solution: Ringer–bicarbonate (calcium-free) solution.
Gas: 93% plus 7% CO_2. Time, 75 min. Temp., 38° C.

Concn. of K^+ (meq./l.) in mucosal and serosal solution	Concn. of luminal or sodium malonate in mucosal and serosal solution, m*M*	Serosal solution, rate of appearance, μM./hr.		% inhibition of glucose transport
		Glucose	Sorbose	
	Luminal			
6.0	Nil	36	3	—
6.0	2	38.8	2	0
6.0	10	35	2.5	0
6.0	40	10.5	2	74
15.6	Nil	55	3	—
15.6	2	45	3.7	18
15.6	10	35	2.8	36
15.6	40	9	2	83
	Sodium malonate			
6.0	Nil	36	3	—
6.0	2	36	4	0
6.0	20	27	3.5	25
15.6	Nil	55	3	—
15.6	2	32	4	42
15.6	10	29	3	47
15.6	20	26	3	53

narcotic in suppressing potassium activation of a phase of glucose metabolism is seen also in the results of experiments on glucose absorption by isolated surviving intestine. Typical values are given in Table III. Here, it will be seen that luminal (phenylethyl barbiturate) exercises little or no inhibition of unstimulated active glucose transport in the isolated surviving intestine until concentrations higher than 10 m*M* are used. When, however, the rate of glucose transport is stimulated by the addition of potassium ions (15.6 meq./liter) the presence of luminal, even at 2 m*M*, brings about a definite inhibition of glucose absorption. At a concentration of 10 m*M* luminal, the rate of absorption of glucose in presence of 15.6 meq./liter K^+ is about identical with that in presence of 6 meq./liter K^+, that is, the presence of the narcotic abolishes the potassium stimulation. Higher concentrations of the barbiturate have similar inhibitive effects on glucose transport independently of the potassium ion concentration. It is evident that, as with glucose respiration in the brain cortex, the barbiturate (luminal) exercises inhibitory effects on a phase of glucose metabolism that is sensitive to the action of potassium ions.

Effects of Sodium Malonate on Glucose Absorption by the Isolated Surviving Guinea Pig Intestine

The effect produced by luminal that has been noted is seen also when sodium malonate is used as inhibitor. It has already been shown (5) that 0.02 *M* sodium malonate inhibits active glucose absorption in the isolated surviving guinea pig intestine by 20%. Ponz and Lluch (19) later found that 0.01 *M* malonate inhibits glucose absorption by rat intestine *in situ* by 26%.

Results presented in Table III show that 2 m*M* sodium malonate, which, when present in a normal Ringer medium, exerts no inhibition of glucose transport in isolated surviving guinea pig intestine, brings about over 40% inhibition of glucose transport when this has been stimulated by the presence of 15.6 meq./liter potassium ions. At a concentration of 20 m*M*, sodium malonate inhibits the rate of unstimulated glucose absorption by 25% but it inhibits the rate of potassium-stimulated glucose absorption by 53%. In effect, the presence of sodium malonate at 2 m*M* abolishes the activating action of potassium ions (15.6 meq./liter) and above this concentration it secures rates of glucose transport that seem to be independent of the potassium ion concentration. Malonate, however, in contrast to phlorizin, dinitrophenol, or luminal, does not lower, at the concentrations investigated, the rate of glucose transport to the passive diffusion rate found under anaerobic conditions. It is already known (12) that the potassium stimulation of brain cortex respiration is inhibited by a concentration of sodium malonate which has relatively little effect on the unstimulated respiration. It was pointed out (9) that this phenomenon indicates that the narcotic-sensitive aspect of brain cortex respiration in the presence of glucose is also highly malonate-sensitive, a fact consistent with the conclusion that the stimulated phase involves the oxidation of carbohydrate through the operation of the citric acid cycle. The same conclusion may also apply to the mechanisms underlying glucose transport in the isolated surviving guinea pig intestine. Both the narcotic (luminal)

and malonate at low concentration abolish the process of potassium stimulation of active glucose transport which is already known to be highly dependent on respiratory processes.

The Effect of Choline on Glucose Absorption by Isolated Surviving Guinea Pig Intestine

In certain experiments devoted to an investigation of the effects of basic organic substances on glucose absorption in the isolated surviving guinea pig intestine, it was found that choline has pronounced inhibitive effects on potassium-stimulated glucose transport. Representative results are shown in Table IV. Choline, at concentrations above 0.5 m*M*, has definite inhibitory effects on unstimulated glucose transport, but the percentage effect is not large, and the rate of transport becomes stabilized at about 20 μM. glucose per hour under the given experimental condition. When the active glucose absorption rate is stimulated by potassium ions (15.6 meq./liter) to a rate of 55 μM./hour, the addition of choline at 0.5 m*M* suppresses the rate to that found in the presence of the normal concentration of potassium (6 meq./liter K^+). Thus the presence of choline can abolish the stimulating action of potassium on glucose transport in isolated surviving guinea pig intestine. It inhibits the rate of glucose absorption but it fails to reduce the rate to the low level shown by passive diffusion of the sugar. It is evident that choline influences a phase of the mechanism of glucose transport in the intestine which is stimulated by the presence of potassium ions.

TABLE IV

EFFECT OF CHOLINE CHLORIDE ON GLUCOSE ABSORPTION BY ISOLATED SURVIVING GUINEA PIG INTESTINE

Mucosal solution: Ringer–bicarbonate (calcium-free) solution. Glucose = 250 mg.%. Sorbose = 200 mg.%.
Serosal solution: Ringer–bicarbonate (calcium-free) solution.
Gas: 93% O_2 plus 7% CO_2. Time, 75 min. Temp., 38° C.

Concn. of K^+ (meq./l.) in mucosal and serosal solution	Concn. of choline chloride in mucosal and serosal solution, m*M*	Serosal solution, rate of appearance, μM./hr.	
		Glucose	Sorbose
Nil	Nil	26	3
Nil	5	19	3
Nil	15	19	3
6.0	Nil	36	3
6.0	0.5	32	3.5
6.0	2	24	2
6.0	5	20	5
6.0	15	21	2
15.6	Nil	55	3
15.6	0.5	30	3.5
15.6	2	26	2

Discussion

A noteworthy feature of the phenomenon of active glucose transport in surviving guinea pig intestine is the fact that the presence of potassium ions accelerates, with relatively low concentrations of glucose, the attainment of the maximum rate of glucose transport found with high concentrations of the sugar (21). The effect of potassium ions is most marked at 15.6 meq./liter K^+ and 14 m*M* glucose. The new facts described in this paper show that the potassium stimulation is abolished by the presence of low concentrations of 2,4-dinitrophenol, phlorizin, luminal, and sodium malonate. This suppression of potassium activation occurs at concentrations of the metabolic inhibitors which have little or no effect on the normal (unstimulated) rate of active transport of glucose and which are lower than those that have been recorded hitherto as effective. The phenomenon is similar to that already known to be associated with the respiration of brain cortex slices where potassium stimulation (which may amount to a doubling of the normal rate) is abolished by low concentrations of malonate and of narcotics such as luminal (9). The high sensitivity of the process to 2,4-dinitrophenol points to the involvement either directly or indirectly of phosphorylation, possibly high energy phosphates, in the process of stimulated active transport of glucose.

A similar conclusion may apply to the results obtained with phlorizin. A marked inhibition of potassium activation of the rate of glucose transport in the isolated guinea pig intestine occurs in the presence of 10^{-5} *M* phlorizin, although this concentration of the inhibitor exercises but little or no effect on the unstimulated rate of glucose transport under our experimental conditions. Jervis *et al.* (10), confirming the results of earlier workers (6, 7, 14, 18) that phlorizin inhibits the rate of glucose transport in the intestine, find a marked effect (e.g. an inhibition of 33%) with 10^{-5} *M* phlorizin on rat intestine *in situ*. It is difficult, however, to assess the significance of their results. They measured the rate of disappearance of glucose from the lumen of the rat intestine and assumed that this rate was the rate of absorption. It has been shown, however, (13) that, in the rat intestine, a considerable breakdown of glucose into lactic acid occurs and it is not yet established whether this process, in the intestine, is effected by phlorizin. It is known that the rate of fermentation of glucose by acetone-dried yeast is inhibited by phlorizin (4), phlorizin being more effective than phloretin, and Lundsgaard (15) later showed that phlorizin inhibits phosphorylations by dry yeast and muscle extracts and glycolysis in brain. Assessment, therefore, of the effects of phlorizin on the rate of glucose transport in rat intestine, by measurement of rates of disappearance from the lumen, involves studies of the effects of phlorizin on rat intestine glycolysis. It is, nevertheless, clear that phlorizin inhibits the rate of glucose transport in intestine at lower concentrations than have been recorded for the inhibition of alkaline phosphatase (10) or mutarotase (11). It is probable, as has been pointed out (10), that phlorizin acts on a more sensitive enzyme system involved in glucose transport in the intestine and

that this process is linked with the potassium stimulation of the rate of glucose transport in the guinea pig intestine.

It should be emphasized that the rate of active galactose transport in guinea pig intestine is also highly sensitive to phlorizin, a result pointing to the presence of a common mechanism for both glucose and galactose. The demonstration (16) that galactose undergoes phosphorylation in the intestine indicates the possibility that this process is involved in galactose transport (see, however, Ref. 22), and that phlorizin exerts its effect on some phase of the phosphorylation system. Active fructose absorption, as shown by the combined transport of fructose and glucose derived from the fructose, is relatively insensitive to phlorizin although a small inhibitive effect is shown when the transport is stimulated by potassium ions. These facts are suggestive of the possibility that the phlorizin effect is located at a phosphorylation step, involved in glucose and galactose transport, which is absent or by-passed in the sequence of events that bring about glucose formation in the intestine from fructose.

Whilst there is a similarity between the effects of phlorizin and 2,4-dinitrophenol insofar as both substances greatly depress, at low concentrations, potassium stimulation of glucose and galactose transport in the intestine, the two substances differ in their relative effects on active fructose transport and active amino acid transport (8). These processes are markedly inhibited by 2,4-dinitrophenol and are almost unaffected by phlorizin. It is, therefore, unlikely that phlorizin exerts its effects by a process similar to that accomplished by 2,4-dinitrophenol.

Although our evidence indicates that phosphorylations are either directly or indirectly involved in the mechanism of sugar transport in the isolated surviving intestine, other evidence does not support the view that hexokinase controls the rate of transport. Mannose, for example, undergoes phosphorylation by a rat intestinal extract at a rate comparable with that of glucose (22), yet mannose is not actively transported. It is also known (2) that 1-deoxyglucose and 6-deoxyglucose are actively transported, but the constitutions of these molecules make it impossible for their phosphorylation by hexokinase to occur. We feel that the facts support the hypothesis that a separate mechanism exists, in the intestine, that controls sugar transport. The concept that separate mechanisms governing transport through cell membranes exist has been considered by various investigators (3, 15, 19, 20, 23). With such a mechanism in the intestine, involvement of phosphorylation is a probability for there is no doubt that the energetics of the cells play a fundamental role in sugar transport.

Acknowledgments

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ABSORPTION OF MIXTURES OF SUGARS BY ISOLATED SURVIVING GUINEA PIG INTESTINE[1]

E. RIKLIS,[2] B. HABER, AND J. H. QUASTEL

Abstract

In the presence of mixtures of glucose and fructose, at a concentration of, or below, 14 m*M*, additive rates of sugar transport by the isolated surviving guinea pig intestine are found. At a concentration of 28 m*M*, or above, the rate of transport of glucose is maximum and no additive rates with mixtures of glucose and fructose at such concentrations are obtained. The rate of transport of sugar from the intestine in the presence of sucrose is that to be expected from the hydrolysis of sucrose to glucose and fructose in the intestine. The increase of concentration of potassium ion to 15.6 meq./liter accelerates the rate of sugar transport from sucrose as well as from glucose–fructose mixtures. The rate of galactose transport by the isolated surviving guinea pig intestine is inhibited 80% in the presence of an equivalent concentration (14 m*M*) of glucose. Fructose, at this concentration, has no inhibitory effect on the rate of galactose transport. Mannose (14 m*M*) and D-glucosamine (14 m*M*) effect 30% inhibitions of galactose transport. Sucrose (14 m*M*) is highly inhibitory (67%) to galactose transport, this probably being due to glucose formation in the intestine. Lactose (14 m*M*) is also apparently inhibitory (46%) to galactose transport, but it is possible that this inhibition is partly due to isotopic dilution by galactose obtained by lactose breakdown. These results are discussed in relation to the phosphorylation hypothesis of sugar transport in the intestine.

Earlier results (4) have shown that the presence of high concentrations of sucrose (e.g. 0.3 *M*) in the lumen of the isolated surviving guinea pig intestine impedes the rates of transport of glucose and fructose from the lumen to the serosal aspect of the intestine. This was considered to be due to hypertonicity in the lumen affecting the rate of the diffusion process in the intestine. The importance of using low concentrations of sugars in the lumen has been stressed in a preceding paper (8) where it was shown that the lower the concentration of fructose in the lumen the higher was the proportion of glucose in the sugar transported. Further work has now been carried out to throw more light on the mechanisms of sugar transport in the intestine by study of the absorption of glucose–fructose mixtures and the effects of sugars on each others' absorption in the isolated surviving guinea pig intestine. Attention has been paid particularly to the effects of sugars on galactose absorption. This paper is concerned with a description of the results of such studies.

Methods and Materials

The experimental procedure adopted is that described fully by Darlington and Quastel (2) and in our preceding papers (8, 9).

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Results

Comparison of the Rates of Glucose and Fructose Absorption from Sucrose with Those from Glucose–Fructose Mixtures in Guinea Pig Isolated Intestine

Typical results showing the rates of appearance of glucose and fructose in the serosal solution when sucrose alone is present in the mucosal solution are given in Table I. It will be seen that the rate of glucose appearance reaches an optimum at a sucrose concentration of about 7 mM, but that the rate of fructose transfer rises gradually as the sucrose concentration is increased. The rate of glucose absorption from a given concentration of sucrose present in the mucosal solution exceeds that from an equivalent concentration of glucose present in the mucosal solution. Representative results showing the variation of rates of glucose transport with changes of glucose and fructose concentrations in the lumen are given in our preceding paper (8). Values recording the rates of glucose transport when sucrose, glucose, or fructose is present alone in the mucosal solution or from a glucose–fructose mixture are shown in Fig. 1. The results are consistent with the conclusion that hydrolysis of sucrose takes place rapidly in the intestine (14), the rate of glucose appearance in the serosal solution being determined by the rates of transport of glucose formed from sucrose and of the glucose derived from fructose obtained by sucrose inversion. In this manner, the phenomenon whereby sucrose is more effective than an equimolar concentration of glucose for promoting glucose transport across the intestine is understood. It is also clear from the results given in Fig. 1 that with low concentrations of glucose and fructose in the

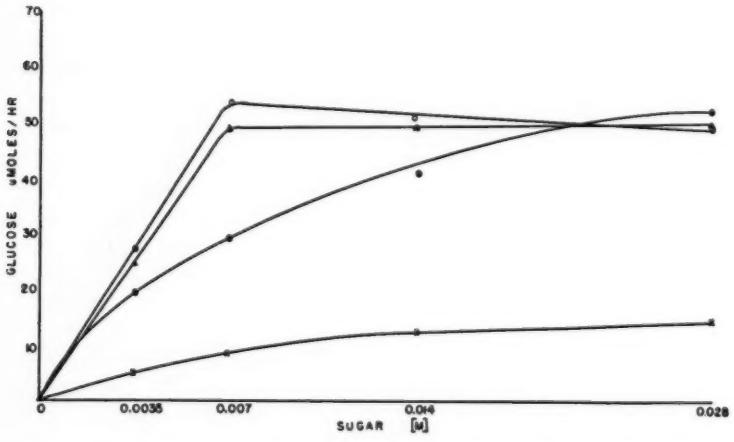

FIG. 1. Rate of transport of glucose in presence of various sugars by isolated surviving guinea pig intestine.
Ordinate: μM./hr. glucose transported into serosal solution.
Abscissa: Initial molar concentration of sugars in mucosal solution.
Gas: 93% O_2 plus 7% CO_2; Ringer–bicarbonate (calcium-free) solution; K^+=6 meq./liter.
○ = Glucose + fructose (equimolar concentration). ● = Glucose.
▲ = Sucrose. × = Fructose.

TABLE I

ABSORPTION OF SUCROSE FROM ISOLATED SURVIVING GUINEA PIG INTESTINE

Mucosal solution: Ringer–bicarbonate (calcium-free) solution. Sucrose concentrations as given below. K^+=6 meq./liter.
Serosal solution: Ringer–bicarbonate (calcium-free) solution. K^+=6 meq./liter.
Gas: 93% O_2 plus 7% CO_2. Time 75 min. Temp. 38°.

Concentration of sucrose in mucosal solution	Serosal solution, rates of appearance (μM./hr.)		
	Glucose	Fructose	Total reducing sugar
0.0035 *M*	24	1	25
0.007 *M*	47.6	2.1	49.7
0.014 *M*	44.5	5.8	50.3
0.028 *M*	45.0	9.8	54.8

lumen, approximately additive rates of glucose transport are obtained from glucose–fructose mixtures. The rates are no longer additive when the glucose concentration in a mixture is about 28 m*M*, this being the concentration above which the rate of glucose transport, under the given experimental conditions, attains a maximum value.

TABLE II

RATES OF TRANSPORT OF GLUCOSE FROM SUCROSE AND SUGAR MIXTURES IN PRESENCE OF VARIOUS POTASSIUM CONCENTRATIONS BY ISOLATED SURVIVING GUINEA PIG INTESTINE

Mucosal solution: Ringer–bicarbonate (calcium-free) solution. Sugar concentrations as stated below.
Serosal solution: Ringer–bicarbonate (calcium-free)solution.
Gas: 93% O_2 plus 7% CO_2. Time 75 min. Temp. 38°.

Mucosal solution	Serosal solution, rates of appearance of glucose (μM./hr.)	
	K^+ in Ringer soln. =6 meq./l.	K^+ in Ringer soln. =15.6 meq./l.
Glucose 3.5 m*M*	21	23
Fructose 3.5 m*M*	5	10
Sucrose 3.5 m*M*	24	38
Glucose 3.5 m*M* + fructose 3.5 m*M*	28	44
Glucose 7 m*M*	30	32
Fructose 7 m*M*	8	16
Sucrose 7 m*M*	47.6	60.4
Glucose 7 m*M* + fructose 7 m*M*	36	58
Glucose 14 m*M*	36	55
Fructose 14 m*M*	12.5	18
Sucrose 14 m*M*	48	56
Glucose 14 m*M* + fructose 14 m*M*	45	54
Glucose 28 m*M*	54	55
Fructose 28 m*M*	15	15
Sucrose 28 m*M*	48	50
Glucose 28 m*M* + fructose 28 m*M*	52	

Effects of Increased Potassium Ion Concentration on Glucose Transport from Sucrose or from Glucose–Fructose Mixtures by Isolated Surviving Guinea Pig Intestine

It has been shown in a preceding paper (8) that increase of the potassium ion concentration in the Ringer solution bathing the intestine to 15.6 meq./liter accelerates the rate of glucose transport at relatively low concentrations of glucose to the maximum value found with high concentrations of glucose. The optimum concentration of glucose at which this accelerating effect is seen is 14 m*M*. Increase of potassium ion concentration does not increase the maximum capacity of the intestinal system to transport glucose, otherwise its activating effect would be seen at higher concentrations of glucose.

Representative results given in Table II show that glucose transport from sucrose is increased by an increase of potassium ion concentration, the effect being quite marked at 3.5 m*M* sucrose. This activating effect is seen also with a glucose–fructose mixture of the same molar concentration. More than additive effects are apparently obtained for the rates of glucose transport from glucose–fructose mixtures in the presence of increased potassium ion concentrations when these rates are compared with those found when each of the sugars is present alone.

When the concentrations of the sugars are increased above 14 m*M*, the rates of glucose transport from a glucose–fructose mixture, or from an equimolar

TABLE III

EFFECTS OF ADMIXTURE OF GLUCOSE AND FRUCTOSE ON SUGAR ABSORPTION BY THE ISOLATED SURVIVING GUINEA PIG INTESTINE

Mucosal solution: Ringer–bicarbonate (calcium-free) solution. Sugar concentrations as stated below. $K^+ = 6$ meq./liter. Volume = 50 ml.
Serosal solution: Ringer–bicarbonate (calcium-free) solution. $K^+ = 6$ meq./liter.
Gas: 93% O_2 plus 7% CO_2. Time 60 min. Temp. 38° C.

	Mucosal solution			Serosal solution		
	Amount present, mg.	Total c.p.m. present	c.p.m./ μg.	Total c.p.m. transferred	mg. sugar transported	μM. sugar transported
Glucose-U-C^{14}	62.5	2.71×10^6	43.3	25×10^4	5.75	32
Fructose-U-C^{14}	62.5	2.60×10^6	41.6	5.1×10^4	1.23	7
Glucose-U-C^{14}	62.5	2.18×10^6	34.8	21.1×10^4	6.0	33
+ fructose	62.5					
Glucose-U-C^{14}	62.5	4.44×10^6	35.5	25.1×10^4	7.07	39
+ fructose-U-C^{14}	62.5					
Fructose-U-C^{14}	62.5	1.56×10^6	25	4.6×10^4	1.84	10.0
+ glucose	62.5					
Glucose-U-C^{14}	125	2.37×10^6	19	13.7×10^4	7.20	40
Fructose-U-C^{14}	130	1.95×10^6	15	3.8×10^4	2.52	14
Glucose-U-C^{14}	125	1.46×10^6	11.7	9.01×10^4	7.7	43
+ fructose	125					
Fructose-U-C^{14}	125	3.4×10^6	27.5	6.44×10^4	2.34	13
+ glucose	125					
Glucose-U-C^{14}	125	5.7×10^6	22.7	21.7×10^4	9.54	53
+fructose-U-C^{14}	125					
Glucose-U-C^{14}	125	2.26×10^6	18	8.53×10^4	4.74	26.5
+ galactose	125					

concentration of sucrose, are less than additive when compared with those of glucose or fructose taken separately. This is explained by the saturation, at high glucose concentrations, of the enzymatic component governing the rate limiting step in the glucose transfer system.

Effects of Admixture of Glucose and Fructose on the Relative Rates of Sugar Absorption by the Isolated Surviving Guinea Pig Intestine

Attempts were made, using radioactive glucose and fructose, to discover whether the presence of one sugar affects the rate of absorption of the other by the isolated surviving guinea pig intestine, although results quoted above (Table II) show additive effects.

Representative results are given in Table III in which rates of sugar absorption are calculated from radioactive counts. They show that, under the given experimental conditions, the presence of fructose (7 m*M* or 14 m*M*) has no impeding effect on the rate of transport of glucose (7 m*M* or 14 m*M*). Moreover, the presence of glucose (7 m*M* or 14 m*M*) has no impeding effect on the rate of transport of sugar derived, in the guinea pig intestine, from fructose (when present at 7 m*M* or 14 m*M*). The results confirm the previous observation that the rates of transport of sugar from mixtures of glucose and fructose (7 m*M* or 14 m*M*) are additive. Each sugar at these concentrations is transported at a rate that is not affected by the presence of the other.

Effects of Sugars on the Rate of Galactose Absorption from Isolated Surviving Guinea Pig Intestine

The effects of the presence of glucose, fructose, mannose, D-glucosamine, sucrose, and lactose on the rate of galactose absorption from isolated surviving guinea pig intestine were studied. Galactose-U-C^{14} was used, the rate of galactose being measured by estimation of the radioactive count in the serosal solution. Typical results are shown in Table IV.

It is evident that glucose (14 m*M*) exercises a powerful inhibition of the rate of galactose transport when this sugar is present at an equimolar concentration. The inhibition amounts approximately to 80%. Some inhibition of glucose transport by galactose (amounting to about 20%–30% at 14 m*M*) occurs as shown by results given in Table III.

The presence of fructose (14 m*M*) exercises no inhibition, within the limits of experimental variation in these experiments, on galactose transport at an equimolar concentration. On the other hand both mannose and D-glucosamine are inhibitors of the rate of galactose transport, their effects being markedly less than that of glucose at the same molar concentration. Inhibitions by mannose and by D-glucosamine are approximately the same (30%) at 14 m*M*.

Sucrose is a potent inhibitor of galactose transport in the isolated surviving guinea pig intestine. The inhibition at 14 m*M* amounts to 67%. It is doubtless due to glucose released from sucrose by hydrolysis in the intestine.

Lactose also exercises an apparent inhibition of galactose transport amounting to about 46% but whether some isotopic dilution takes place owing to possible galactose release from the lactose has still to be ascertained.

TABLE IV

EFFECTS OF SUGARS ON GALACTOSE ABSORPTION BY THE ISOLATED SURVIVING GUINEA PIG INTESTINE

Mucosal solution: Ringer–bicarbonate (calcium-free) solution. Sugar concentrations as stated below. Volume = 50 ml.
Serosal solution: Ringer–bicarbonate (calcium-free) solution.
Gas: 93% O_2 plus 7% CO_2. Time 60 min. Temp. 38° C.

	Mucosal solution			Serosal solution		
	Amount present, mg.	Total c.p.m. present	c.p.m./ μg. galactose	Total c.p.m. transferred	mg. galactose transported	μM. galactose transported
Galactose-U-C^{14}	125	7.0×10^5	5.6	4.15×10^4	7.42	41
Galactose-U-C^{14}	125	1.37×10^6	11	6.19×10^4	5.63	32
Galactose-U-C^{14}	125	1.33×10^6	10.6	7.95×10^4	7.5	41.6
+ fructose	125					
Galactose-U-C^{14}	125	1.46×10^6	11.6	6.93×10^4	5.95	35
+ fructose	125					
Galactose-U-C^{14}	125	1.08×10^6	8.7	5.79×10^3	0.67	4
+ glucose	125					
Galactose-U-C^{14}	125	1.02×10^6	8.2	1.75×10^4	2.13	12
+ glucose	125					
Galactose-U-C^{14}	125	1.26×10^6	10.1	1.33×10^4	1.31	7
+ glucose	125					
Galactose-U-C^{14}	125	1.57×10^6	12.6	6×10^4	4.76	26.5
+ mannose	125					
Galactose-U-C^{14}	125	8.28×10^5	6.6	2.88×10^4	4.37	24.5
+ mannose	125					
Galactose-U-C^{14}	125	1.58×10^6	12.6	5.49×10^4	4.36	24.5
+ D-glucosamine	150					
Galactose-U-C^{14}	125	2.07×10^6	16.5	8.53×10^4	5.16	28
+ D-glucosamine	150					
Galactose-U-C^{14}	125	8.6×10^5	6.9	2.85×10^4	4.13	23
+ lactose	250					
Galactose-U-C^{14}	125	2.04×10^6	16.3	4.81×10^4	2.96	16
+ lactose	250					
Galactose-U-C^{14}	125	1.39×10^6	11.1	2.53×10^4	2.28	13
+ sucrose	250					
Galactose-U-C^{14}	125	8.6×10^5	6.9	1.3×10^4	1.89	10.5
+ sucrose	250					

Discussion

It is evident from the observations which have been recorded above that at concentrations up to 14 m*M* neither glucose nor fructose affects each other's rate of absorption by the isolated surviving guinea pig intestine. Additive rates of transport take place at such concentrations. The rates of absorption of sugars, when sucrose alone is present in the lumen of the intestine, are those to be expected if the sucrose undergoes hydrolysis in the intestine. The fact that the rate of glucose transport from sucrose is greater than from an equimolar concentration of glucose is due to conversion of fructose, derived from sucrose, into glucose. At concentrations of glucose higher than 28 m*M*, the rate of sugar transport, under the given experimental conditions, attains a maximum. Additive effects are not obtained with mixtures of glucose and fructose at or above this concentration. It is possible that inhibition of

fructose phosphorylation by glucose (10) or by glucose-6-phosphate (11, 12) may be partly responsible for lack of additive effects on rates of glucose transport in presence of mixtures of glucose and fructose at relatively high concentrations (14 m*M*) of the sugars.

The effects of sugars on the rate of galactose transport are of importance in connection with the possible involvement of phosphorylation processes in sugar transport in the intestine.

It is known that active galactose transport occurs in the intestine of guinea pig (2,4) and rat (3). Results given in our preceding paper (9) show that the rate of galactose transport in the isolated guinea pig intestine is suppressed by phlorizin, the sensitivity to phlorizin being increased by the presence of 15.6 meq./liter potassium ions which accelerate the rate of galactose transport under aerobic conditions. These phenomena are similar to those occurring with glucose transport in the isolated guinea pig intestine and point to a similarity of the mechanisms of transport of glucose and galactose. Fisher and Parsons (3) have shown, with isolated rat intestine, mutual effects of glucose and galactose on their absorptive rates. The rate of absorption of galactose, when present at a concentration of 0.5%, is halved in the presence of an equal concentration of glucose, whilst that of glucose is diminished by about 15% by the presence of the galactose. Mutual effects of glucose and galactose on their absorptive rates have been described by Cori (1) and Westenbrink and Middlebeek (13). Nirenberg and Hogg (7) have demonstrated an inhibitory effect of galactose on fructose breakdown but not on glucose breakdown in intact Ehrlich ascites tumor cells. There is no inhibition, however, of fructose breakdown in Ehrlich ascites cell homogenates. They find that galactose at a concentration high enough to give 80–90% inhibition of fructose utilization in the cellular system has no effect on the hexokinase of Ehrlich ascites cells. Our results, using radioactive galactose, show that the presence of glucose at an equimolar concentration (14 m*M*) exercises 80% inhibition of the rate of galactose transport. Sucrose, at the same molar concentration, is almost equally effective and doubtless this may be explained by the formation of glucose from sucrose. Fructose, however, at the same concentration seems to be without inhibitory effect. This fact, when correlated with the lack of inhibitory action of phlorizin on fructose conversion to glucose (9), suggests that an initial step in fructose active absorption differs from that involved in glucose or galactose absorption.

Although Sols (10) could find no evidence of galactose phosphorylation in a rat intestinal mucosa homogenate, it has been reported (6) that galactose 1- and 6-phosphates accumulate in the rat small intestine during galactose absorption. In view of this latter evidence and the fact of high sensitivity of galactose transport to phlorizin, there seems to be a likelihood that galactose phosphorylation is involved in galactose transport. The inhibition of galactose transport by glucose would be explained then by an interference of glucose or of glucose phosphate with the synthesis or breakdown of galactose phosphate in the

isolated surviving guinea pig intestine. Our results also show definite inhibitions of the rate of galactose transport by the presence of D-glucosamine or mannose at equivalent concentration. Phosphorylation of these sugars which is known to occur with hexokinase (5, 10) may give rise to phosphate esters in the guinea pig intestine that inhibit galactose phosphorylation or galactose phosphate breakdown, thus inhibiting its transport.

The evidence, as a whole, embracing the results of studies on potassium ion activation of rates of sugar transport (8), inhibitions by 2,4-dinitrophenol (9), by malonate (9), and by phlorizin (9), and the mutual effects of mixture of sugars on their rates of transport, make it likely that phosphorylation reactions are involved in the mechanism of sugar transport, in the intestine but do not necessarily control the rate of transport. It has been suggested in a preceding paper (9) that an enzymatic mechanism other than hexokinase exists in the intestine that controls the rate of sugar transport. With this mechanism phosphorylations are either directly or indirectly connected.

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